

Australia

Patents Act 1990

NOTICE OF ENTITLEMENT

I, John Gordor Hinde, of Spruson & Ferguson, St Martins Tower, 31 Market Street, Sydney, New South Wales 2000, Australia, being the patent attorney for the Applicant(s)/Nominated Person(s) in respect of Application No 36001/93 state the following:-

The Applicant(s)/Nominated Person(s) has/have entitlement from the actual inventor(s) as follows:-

The Applicant(s)/Nominated Person(s) is/are the assignee(s) of the actual inventor(s).

The Applicant(s)/Nominated Person(s) is/are entitled to rely on the application(s) listed in the Declaration under Article 8 of the PCT as follows:

The Applicant(s)/Nominated Person(s) is/are the assignee(s) of the basic applicant(s).

The basic application(s) listed on the Declaration under Article 8 of the PCT is/are the first application(s) made in a Convention country in respect of the invention.

DATED this 21st day of July 1994

.....
John Gordon Hinde

IRN: 277530

INSTR CODE: 57700



AU9336001

(12) PATENT ABRIDGMENT (11) Document No. AU-B-36001/93
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 680405

(54) Title
NUCLEOZYMES

International Patent Classification(s)
(51)⁵ C12N 015/11 A61K 031/70

C07H 021/00 C12P 019/34

(21) Application No. : 36001/93

(22) Application Date : 28.01.93

(87) PCT Publication Number : WO93/15187

(30) Priority Data

(31) Number	(32) Date	(33) Country
829729	31.01.92	US UNITED STATES OF AMERICA

(43) Publication Date : 01.09.93

(44) Publication Date of Accepted Application : 31.07.97

(71) Applicant(s)
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

(72) Inventor(s)
NASSIM USMAN; ROBERT J CEDERGREN; JEAN-PIERRE PERREAULT; JING-HUA YANG;
ALEXANDER RICH

(74) Attorney or Agent
SPRUSON & FERGUSON, GPO Box 3898, SYDNEY NSW 2001

(57) Claim

1. An enzymatic nucleic acid molecule having catalytic activity comprising a plurality of catalytically critical sites and a plurality of catalytically non-critical sites the enzymatic nucleic acid molecule including a catalytic centre comprising the catalytically critical sites, and a plurality of catalytically non-critical sites, wherein the catalytically critical sites are ribonucleotides or nucleic acid analogues capable of participating in the catalytic activity of the enzymatic nucleic acid molecule and a majority of the catalytically non-critical sites in the catalytic centre are deoxyribonucleotides, the enzymatic nucleic acid molecule having an increased resistance to degradation compared to an all RNA counterpart.

**CORRECTED
VERSION***

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION



36001/93

pages 1/6-6/6, drawings, replaced by new pages 1/3-3/3; due
INTERNATIONAL to late transmittal by the receiving Office

T COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 9/00, A61K 31/70 C07H 21/00, C12P 19/34		A1	(11) International Publication Number: WO 93/15187 (43) International Publication Date: 5 August 1993 (05.08.93)
(21) International Application Number: PCT/US93/00833 (22) International Filing Date: 28 January 1993 (28.01.93) (30) Priority data: 07/829,729 31 January 1992 (31.01.92) US (71) Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 28 Carleton Street, Cambridge, MA 02139 (US). (72) Inventors: USMAN, Nassim ; 3 Lawrence Street, Boston, MA 02116 (US). CEDERGREN, Robert, J. ; 4675 Christophe Colomb, Montreal, Quebec H2J 3G7 (CA). PERREAULT, Jean-Pierre ; 3291 Hanna Street, Rawden, Quebec J0K 1S0 (CA). YANG, Jing-Hua ; 2676 Monpetit Avenue, Montreal, Quebec H3T 1J6 (CA). RICH, Alexander ; 2 Walnut Avenue, Cambridge, MA 02116 (US).		(74) Agent: GATES, Edward, R ; Wolf, Greenfield & Sacks, 600 Atlantic Avenue, Boston, MA 02210 (US). (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: NUCLEOZYMES			
(57) Abstract Nucleozymes containing ribonucleotides and deoxyribonucleotides or nucleic acid analogues are described herein. The nucleozymes have catalytic activity and are significantly more resistant to degradation than their all-RNA ribozyme counterparts. Also described are methods for preparing the nucleozymes along with methods of using nucleozymes, e.g., as therapeutic agents.			

68 0 4 0 5

* (Referred to in PCT Gazette No.30/1993, Section II)

-1-

NUCLEOZYMES

Field of the Invention

This invention relates to nucleozymes, mixed nucleic acid polymers having catalytic activity. The invention also relates to methods of preparing and using nucleozymes.

Background of the Invention

Proteins were the only known catalysts of cellular reactions until the discovery of RNA catalysts (ribozymes). In some instances, the folded structure of a ribozyme catalyses a cleavage reaction on another part of the same molecule (cis-reaction). In other instances (trans-reaction), the ribozyme may also act as a catalyst on another RNA or DNA molecule (substrate) by cleaving or ligating pieces of the substrate without changing itself in the process. (Zaug et al. Science, Vol. 231, pp. 470-75, 1986; Cech Science, Vol. 236, pp. 1532-39, 1987).

A well-characterized example of a ribozyme is the self-splicing Group I intron from the nuclear rRNA of Tetrahymena thermophila. An intron is an intervening sequence in a eukaryotic gene which does not encode a protein or in rare cases encodes a different protein. Introns are transcribed along with coding sequences (exons) to produce precursor RNA. The introns are removed from the precursor RNA and the exons are ligated by RNA cleaving and splicing steps. The Group I intron or ribozyme of T. thermophila catalyzes its own removal from the precursor RNA molecule. (Kruger et al. Cell

- 2 -

31:147-157, (1982); Zaug et al. (1986)). The self-splicing ribozyme catalyzes a variety of phosphodiester transfer reactions. The ribozyme can act as a ribonuclease, ligase, phosphotransferase, acid phosphatase, polymerase and RNA restriction endonuclease (Zaug, A.J., et al., Science 231:470-475 (1986); Zaug, A.J., et al., Nature 324:429-433 (1986); Zaug, A.J., et al., Biochemistry 25:4478-4482 (1986); Been, M.D., et al., Science 239:1412-1416 (1988); Doudna et al., Nature 339:519-522 (1989); all incorporated by reference herein).

The "hammerhead" and "hairpin" ribozymes also have been studied and described (Perreault et al., Nature 344:565-567 (1990); Perreault et al., Biochemistry 30:4020-25 (1991); Yang et al., Biochemistry 29:11156-60 (1990); Chowrira et al., Biochemistry 30:8518-22 (1991); Uhlenbeck Nature, 328:596-600 (1987)). The hammerhead ribozyme forms a stem loop secondary structure to form the catalytically active molecule. The hairpin ribozyme has a structure resembling a hairpin.

Rossi et al. WO91/03162 describe a modified hammerhead ribozyme comprising a ribozyme catalytic centre and deoxyribonucleotides in place of ribonucleotides external to the catalytic centre. Pieken et al. Science 253: 314-317 (1991) describe modified hammerhead ribozymes incorporating 2'-fluoro- and 2'-amino- nucleotide analogues which exhibit enhanced stability to degradation in rabbit serum.

Although ribozymes are intriguing molecules, their use for in vivo applications is limited if not precluded. The all-RNA molecules are susceptible to degradation from enzymes (RNAses) present in vivo. There presently is no way known to inventors for delivering such molecules to the intended site in an active form.



AMENDED SHEET

- 3 -

Summary of the Invention

The present invention is based on the discovery that ribozymes have catalytically critical sites and that it is not necessary to have an all-RNA molecule to have catalytic activity. The ribozyme like molecules of the invention or "nucleozymes" have ribonucleotides or nucleic acid analogues (hereinafter NAAs) at catalytically critical sites and NAAs or deoxyribonucleotides at non-catalytically critical sites. The preferred nucleozymes have ribonucleotides at catalytically critical sites. Nucleozymes have catalytic activity on the same substrates as their ribozyme counterparts.

The nucleozymes of the present invention thus essentially are modified ribozymes having at least a portion, or all, of the ribonucleotides replaced with deoxyribonucleotides or NAAs. The nucleozymes are significantly more resistant to degradation than their all-RNA ribozyme counterparts because the chemicals or enzymes present in vivo do not recognize the nucleic acid internucleotide bonds. The resistance can be to either enzymatic or chemical degradation. Preferably, a majority of the ribonucleotides of the ribozyme are replaced with deoxyribonucleotides or NAAs. The stability of the nucleozymes allows them to be useful as therapeutic agents whereas ribozymes would be cleaved and rendered inactive by enzymes, e.g. RNAses, present in vivo.

- 4 -

The nucleozymes of the present invention are chimeric nucleic acid polymers having catalytic activity due to and preferably optimized by the presence of RNA or a NAA at a catalytically critical site. The present invention provides chemistry which allows synthesis of the chimeric polymers and the determination of catalytically critical sites. The sites may be determined by varying the location of deoxyribonucleotides in a chimeric polymer and determining the locations responsible for or related to the chimeric polymer's ability to catalyze.

The present invention also pertains to a method for making a chimeric polymer. The polymers are made by phosphitylating protected ribonucleotides or NAAs units under conditions to form substantially pure-protected phosphoramidites or synthons of a single isomer. The protected phosphoramidites are coupled to each other forming a protected chimeric nucleic acid chain. The protecting groups are removed from the chimeric nucleic acid chain under conditions which completely deprotect the polymer.

The method of the present invention alleviates problems associated with a known prior art method for preparing chimeric RNA/DNA polymers (Perreault et al. Nature 344:565-567 (1990); Wu et al. Journal of the American Chemical Society 111:8531-33 (1989)). The prior art synthetic method for making chimeric polymers had problems with the migration of the protecting groups during the phosphitylating step, difficulty in removing the protecting groups and also

- 5 -

has problems resulting from the process of removing the protecting groups in the deprotection step. The former problem results in the production of monomer units having protecting groups in an undesired position. The latter problem results, in many cases, in a) nucleotide modification, b) phosphodiester linkage isomerization, and c) to retention of a substantial amount of protecting groups on the polymer resulting in a non-functional polymer. The first problem was overcome in the present invention by selecting a catalyst capable of minimizing migration of protecting groups, e.g., a combination of 2,4,6-collidine and N-methylimidazole. The problem in removing the protecting groups was eliminated by deprotecting the mixed polymer in the presence of ethanolic ammonia.

The present invention also pertains to methods of using the nucleozymes. The nucleozymes may be used to perform the same catalytic functions as their all-RNA ribozyme counterparts. For example, a nucleozyme may be used as a ribonuclease, ligase, phosphotransferase, acid phosphatase, polymerase, or an RNA restriction endonuclease. The nucleozymes may be used to selectively cleave and ligate substrates by contacting the substrates with a nucleozyme such that the nucleozyme targets a specific sequence in the substrate for cleavage or ligation. The nucleozymes may be used as polymerases to polymerize the production of an oligoribonucleotide or an oligodeoxyribonucleotide. The nucleozymes also may be used in place of antisense RNA technology.

- 6 -

The nucleozymes also may be used as therapeutic agents introduced in vivo due to their resistance to chemical and enzymatic degradation. The nucleozymes may be used, for example, in a method for treating a subject for a retrovirus associated disease, e.g., human immunodeficiency virus (HIV). The method involves administering a therapeutically effective amount of at least one nucleozyme to the subject such that the nucleozyme cleaves the RNA genome of the retrovirus rendering it inactive. A plurality of nucleozymes also may be administered if it is desirable to target more than one sequence in the RNA genome.

A nucleozyme may be provided in a pharmaceutical composition. The pharmaceutical composition would include at least one nucleozyme and a pharmaceutically acceptable carrier.

It is an object of the present invention to provide a nucleozyme capable of maintaining its catalytic properties in vivo.

It is an object of the present invention to provide a chimeric nucleic acid polymer having catalytic activity.

It is yet another object of the present invention to provide a method for preparing chimeric polymers which are free of protecting groups and undesired isomeric side products.

It is yet another object of the present invention to provide a homogenous chimeric polymer.

- 7 -

Brief Description of the Drawings

FIG 1 depicts a structure of a hammerhead nucleozyme.

FIG 2 is a table listing nucleozymes of the present invention indicating the positions of ribonucleotides in the nucleozymes. The position designations correspond to the structure depicted in FIG 1.

FIGs 3 and 4 are photographs of a gel demonstrating the cleavage of a 5'-³²P-labeled RNA substrate. The designation "RNA" is the all-RNA containing ribozyme and the nucleozymes are as abbreviated in FIG 2.

FIG 5 is a photograph of a gel demonstrating the cleavage of a radioactive RNA substrate by nucleozymes of the present invention containing methoxy substituted NAAs. The nucleozymes are abbreviated as in FIG 2.

FIGs 6 and 7 are graphs depicting the stability of nucleozymes compared to the stability of the all-RNA ribozyme counterpart after exposure to both RNase A (6) and a yeast cellular extract (7).

Detailed Description

The nucleozymes of the present invention have catalytic activity. Nucleozymes essentially are modified ribozymes preferably having at least one ribonucleotide or nucleic acid analogue (NAA) at a catalytically critical site(s) and deoxyribonucleotides or NAAs at non-critical sites.

- 8 -

The term "nucleozyme" is intended to include catalytic chimeric polymeric chains containing ribonucleotides and deoxyribonucleotides and/or nucleic acid analogues.

A ribozyme is an all-RNA containing molecule capable of being a biological catalyst. Ribozymes are art recognized and examples of such molecules include the "hammerhead" or "hairpin" ribozymes.

The term "catalytic activity" is intended to include many types of catalysis. For example, the catalytic activity could be that of a ribonuclease, ligase, phosphotransferase acid, phosphatase, polymerase, and RNA restriction endonuclease.

The term "nucleic acid analogue" (NAA) is intended to include analogues which are structurally similar to ribonucleotides or deoxyribonucleotides and are capable of being monomer units in a polymer capable of hybridizing with DNA or RNA. The analogue may impart properties to a polymeric chain which differ from those of a nucleotide but the analogue is capable of being a monomer unit in a polymeric chain. The NAA may impart resistance to chemical or enzymatic degradation to the chimeric polymer. NAAs may be selected for their structural conformation if a particular conformation is desired for the polymer. A NAA which is structurally similar to a ribonucleotide may be positioned at a catalytically site if the NAA is capable of participating and/or attaining the desired catalytic activity. Preferably, if the NAA is positioned at a

WO 93/15187

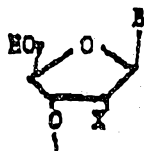
- 9 -

PCT/US93/00833

catalytically critical site it has a formula as depicted in Formulas I-IV below wherein X is a good coordinating ligand with divalent metal ions, eg., Mg^{+2} .

A nucleotide analogue may contain a heterocyclic ring as depicted in the formulae I-IV below or may be acyclic as shown in Formula V below. The preferred heterocyclic ring containing analogues of the present invention have three hydroxy substituents, alkoxy substituents or combinations thereof.

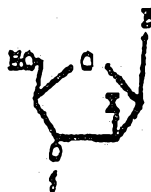
Formula (I)



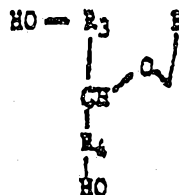
Formula (III)



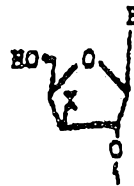
Formula (II)



Formula (V)



Formula (IV)



In the above formulae, B is a base. The base may be substituted or unsubstituted. Examples of bases include adenine, cytosine, guanine, uracil,

2-aminopurine, hypoxanthine, imidazole, $R_1-C(=O)-O-$,

- 10 -

R_1 -SH-, and R_1 -NH₂-. The term "base" is art-recognized and one of ordinary skill in the art would know which bases are useful in the present invention. X is selected from the group consisting of -OR₁, F, -R₂OH, -NH₂, -R₂NH₂, -Br, -R₂Br and -R₂F wherein R₁ is a lower alkyl group and R₂-R₄ are a lower alkyl hydrocarbon chains. The term "lower alkyl" is intended to include from one to six carbons, more preferably one to three carbons and most preferably a single carbon atom. For example, the most preferred X is methoxy.

B may be protected during the synthesis process. The protecting groups may be the conventional groups typically used in oligonucleotide synthesis processes e.g., N⁶-benzoyl for adenine, N⁴-benzoyl for cytosine, N²-isobutyryl for guanine, and N²-benzoyl for 2-aminopurine. Other useful protecting groups include phenoxyacetyl (PAC) and t-butoxyacetyl (TAC). One of ordinary skill in the art would know which protecting groups are appropriate for a particular base.

The NAAs capable of being positioned at a catalytically critical site may be determined by one of ordinary skill in the art using the following screening process. The catalytically critical sites of a particular ribozyme may be determined by positioning deoxyribonucleotides(s) at various locations within the ribozyme and evaluating the chimeric polymer's catalytic activity. After determining the locations of the catalytically

- 11 -

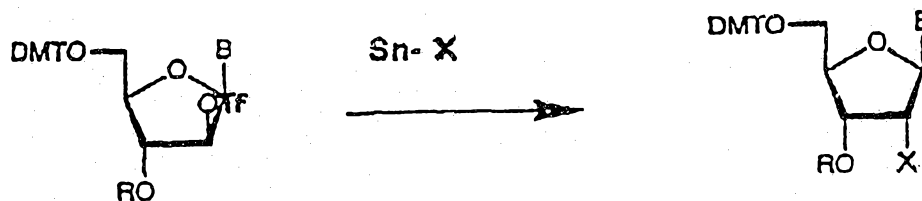
critical sites, NAAs may be substituted for the deoxyribonucleotide(s) and the chimeric polymer's catalytic activity is again evaluated. If the NAA containing chimeric polymer possesses catalytic activity then the NAA is suitable for positioning at a catalytically critical site.

The term "synthon" is intended to include the fully protected monomer units (phosphoramidites) used to assemble the nucleic acid analogues of a chimeric polymer chain. The term "nucleic acid analogue" is used to describe the units when polymerized as part of a chimeric polymer chain.

The term "chimeric polymer" is intended to include polymers containing at least two different types of monomer units, e.g., RNA, DNA, or NAA. For example, a chimeric polymer may include RNA/DNA, RNA/NAA, or DNA/NAA polymeric chains. It should be understood that the linkages between the building units of the polymeric chain may be linkages capable of bridging the units together for either in vitro or in vivo. For example, the linkage may be a phosphorous containing linkage, e.g., phosphodiester or phosphothioate, or may be a nitrogen containing linkage, e.g., amide. It should further be understood that the chimeric polymer may contain non-nucleotide spacer molecules along with its other nucleotide or analogue units. Examples of spacer molecules which may be used are described in Nielsen et al. Science, 254:1497-1500 (1991), the contents of which are expressly incorporated by reference.

- 12 -

The analogues described above may be prepared using synthetic methods capable of attaching the desired moieties at the selected positions. Other possible analogues will be apparent to one of ordinary skill in the art. Deoxyribonucleotides are readily available and may be purchased from a variety of sources. The alkyl substituted heterocyclic ring containing analogues may be synthesized by first reacting the moiety being modified with arabino triflates (trifluoromethane sulfonates) and second displacing the arabino triflates with an appropriate organotin compound selected on the basis of the desired moiety. A schematic of this reaction is depicted below:



This synthesis scheme may be used to prepare analogues wherein X is selected from the group consisting of $-OR_1$, $-R_2OH$, R_2F , $-RBr$, and $-RNH_2$. One of ordinary skill in the art would know how to synthesis the halogenated analogues (X is $-F$ or $-Br$) and the amino substituted analogues (X is

- 13 -

-NH₂). These analogues may be synthesized as described by Williams et al., Biochemistry, 30:4001-4009 (1991), Doerr et al., J. Org. Chem., 32:1462 (1967), Mengel et al., Angew. Chem., 90:557 (1978), or Coddington et al., J. Org. Chem., 29:558 (1964), the contents of each of the references are hereby expressly incorporated by reference.

The acyclic nucleic acid analogues may be prepared by reacting a protected acyclic molecule with a diol. Reaction processes which may be used are described in Durand et al., Nucleic Acid Research, 18:6353 (1990); Seela et al., Nucleic Acid Research, 15:3113-3124 (1987); Cload et al., JACF, 113:6324-6326 (1991), the contents of each reference is hereby expressly incorporated by reference.

The nucleozymes have catalytically critical site(s) at which a ribonucleotide or NAA is necessary for the nucleozyme to have the desired level of catalytic activity. The term "catalytically critical site" is intended to include sites which, if altered from a ribonucleotide or a NAA to a deoxyribonucleotide, substantially reduces or even eliminates catalytic activity. A substantial reduction in catalytic activity would be that reduction which limits the usefulness of the nucleozyme as a catalyst in vitro or in vivo. The catalytically critical sites may be determined for each nucleozyme. Catalytically critical sites can be determined by preparing a variety of chimeric polymers using the chemical techniques described

- 14 -

herein and comparing the catalytic activity of the chimeric test polymers. The catalytically critical sites in the all-RNA containing ribozyme counterparts are determined by selecting sites believed to be involved in catalysis and inserting a deoxyribonucleotide at the particular site. If the chimeric polymer does not have the same or substantially the same catalytic activity as the all-RNA counterpart, then the selected site is presumed to be a catalytically critical site. The hammerhead nucleozyme has four catalytically critical sites which are the G9, G12, A13 and A29 positions for the sequence depicted in FIG 1.

The nucleozymes of the present invention are of a size capable of being synthesized using the chemistry described herein. Preferably, the nucleozymes have less than about 100 total building units, more preferably, less than about 80 building units, even more preferably, less than about 70 building units, and most preferably less than about 50 building units. Some nucleozymes may even have less than about 20 building units. The preferred nucleozyme is modeled after the hammerhead ribozyme, the catalytic portion of which has 35 building units. The term "building unit" is intended to include ribonucleotides, deoxyribonucleotides, or synthons.

The nucleozymes of the present invention are modified ribozymes having at least a portion of the ribonucleotides replaced with deoxyribonucleotides or

- 15 -

NAAAs. The modified ribozymes or nucleozymes are significantly more resistant to degradation than the all-RNA counterparts. The degradation may be either enzymatic or chemical degradation. The language "significantly more resistant to degradation" is that resistance which allows the nucleozyme to remain largely intact for an extended period of time relative to its all-RNA counterpart. Preferably it has a resistance which allows it to be administered for in vivo applications.

Resistance to enzymatic degradation may be resistance to enzymes present in vivo, e.g. RNases such as RNase A. FIGS. 6 and 4B are graphs depicting the relationship between the percentage of the nucleozyme being intact at a particular RNase A (FIG. 6) or yeast cellular extract (FIG. 7) concentration. The data set forth in FIGS. 6 and 7 was obtained using the following procedure. A sample of 0.001 pmol of 5' labeled nucleozyme or ribozyme was incubated with 0.5 mg of carrier tRNA in 50 mM Tris-HCl (pH 7.4) and 10 mM Mg^{+2} and different concentrations of RNase A or yeast extract ten minutes for A, thirty minutes for extract. The reactions were stopped by the addition of 20 mM EDTA and loaded and analyzed on 15% PAGE in 7M urea. The yeast extract was prepared from a 1 ml culture of yeast strain BWG2-9A grown to late-lag phase, harvested and washed with 25 mM sodium phosphate buffer (pH 7.8). The pellet was suspended in 100 μ l of the same buffer and sonicated for 20 seconds

- 16 -

(60W). After centrifugation for five minutes in an Eppendorf centrifuge, the supernatant was used directly after appropriate dilutions and incubations with the nucleozymes and the all-RNA ribozyme.

As shown in FIG. 6, the nucleozymes were at least about 75% intact at an RNase A log concentration of -1.5 and at least about 80% intact at an RNase A log concentration of -2.5. Preferably, the nucleozyme is at least about 90% intact at both concentrations. The difference in stability between the nucleozymes and ribozyme is even more apparent when the enzyme is a yeast cellular extract (FIG. 7). The nucleozymes were at least 90% intact at all of the tested concentrations.

The chemical degradation for purposes of this invention is intended to include resistance to chemicals present in vivo and in vitro. The resistance may be to alkaline hydrolysis, e.g., sodium hydroxide and water.

The preferred nucleozymes of the present invention are modified ribozymes having a majority of the ribonucleotides replaced with deoxyribonucleotides or NAAs. At least one of the units is a deoxyribonucleotide and preferably most of the units are deoxyribonucleotides. The nucleozymes more preferably, have at least about 75%, even more preferably at least about 85%, most preferably at least about 90% of their ribonucleotides replaced with deoxyribonucleotides or NAAs. The nucleozyme also may be made up entirely of NAAs or a combination of NAAs and deoxyribonucleotides.

- 17 -

The present invention also pertains to a method of making a chimeric polymer. The method is similar to that described by Scaringe et al. to produce an all-RNA polymer. (Nucleic Acid Research Vol. 18, No. 18, 5433-41 (1990)), the contents of which is expressly incorporated by reference. The method includes the steps of phosphitylating protected RNA, DNA or NAA units under conditions which minimize migration of the protecting group forming isomerically pure protected phosphoramidites. The protected RNA or synthons may have the desired moieties protected with the protecting groups capable of surviving the phosphitylation and coupling steps. Examples of such groups include conventional DNA protecting groups such as 5'-O-DMT, N-Bz (Ade and Cyt), N-iBu (Gua), β -cyanoethyl for phosphate, TB DMS for 2'-hydroxyl.

The term "isomerically pure protected phosphoramidites" is intended to include phosphoramidite preparations free of a substantial amount of undesired isomers of the phosphoramidites. A substantial amount is that amount which would substantially interfere or impede with the preparation's ability to be used in forming a mixed polymeric chain. For example, a consideration in the chemical synthesis of a ribonucleotide phosphoramidite is contamination of the desired 2'-O-protecting group-3'-O phosphoramidite with the undesired 3'-O- protecting group -2'-O-phosphoramidite. Syntheses performed with the

- 18 -

latter lead to oligonucleotides having 5'-2' linkages. Isomerically pure protected phosphoramidites of the present invention lead to oligonucleotides which are free of such undesired linkages.

The method of the present invention also involves coupling the protected phosphoramidites together forming a protected chimeric polymeric chain. The coupling can be done using well-known chemical techniques known to one of ordinary skill in the art. Preferably the coupling is done on an automated synthesizer.

After the chimeric polymeric chain is formed, the protecting groups now can be removed from the chain under conditions which completely deprotect the polymer. Also, nucleotide base modification and/or phosphodiester linkage isomerization of the chain now can be minimized during the deprotection step.

The prevention of migration of the protecting groups in the phosphitylation step can be accomplished by phosphitylating in the presence of a catalyst selected to minimize migration. An example of such a catalyst is the combination of 2, 4, 6-collidine and N-methylimidazole.

The invention permits removal of the protecting groups in a manner that is complete and that minimizes nucleotide base modification and/or phosphodiester linkage isomerization. Complete removal of protecting groups includes substantially complete removal where a polymeric chain may have a

- 19 -

small number of protecting groups still attached which do not effect the polymeric chain's intended catalytic function. The deprotection step is accomplished by deprotecting the polymer in the presence of an agent capable of minimizing such effects. An example of such an agent is ethanolic ammonia.

The method also allows the production of a homogeneous RNA/DNA polymer free of undesired isomeric products. The term "free" is intended to include substantially free wherein a small amount of protecting groups or undesired isomers are present as long as the amount does not interfere or impede the polymer's function. The minimization of nucleotide base modification is intended to include that modification which would effect a polymeric chain's intended catalytic function. Minimizing phosphodiester linkage isomerization, when used in connection with a molecule, means preventing that degree of isomerization which would, adversely affect the molecule's intended catalytic function. Minimizing phosphodiester linkage isomerization when used in connection with a preparation, means preventing that degree of isomerization which would substantially affect the preparation's abilities to be used for its intended catalytic functions.

The present invention also pertains to methods for using the nucleozymes. The nucleozymes may be used for any method in which a ribozyme presently may be used. For example, the nucleozyme may be used to

- 20 -

selectively cleave an RNA substrate or to ligate two pieces of RNA together. When cleaving a substrate, the RNA substrate is contacted with at least one nucleozyme which targets a specific sequence in the substrate for cleavage. A plurality of nucleozymes also may be used in a cleavage process.

The nucleozymes of the present invention also may be used to polymerize an oligonucleotide molecule. The oligonucleotide may contain ribonucleotides, deoxyribonucleotides, and analogues of deoxy- or ribonucleotides. A template is contacted with a population of the appropriate nucleotide monomer units and a nucleozyme under conditions which allow an oligonucleotide complementary to the template to form. The template preferably is attached to a support.

The nucleozymes of the present invention also may be used for therapeutic methods due to their stability in vivo. The nucleozymes may be used, for example, to treat a subject for a retrovirus associated disease. A therapeutically effective amount of at least one nucleozyme is administered to a subject such that the nucleozyme(s) cleaves the RNA genome of the retrovirus or the viral mRNA rendering it inactive.

A retrovirus associated disease is intended to include diseases involving retroviruses. Retroviruses have an RNA genome making them susceptible to cleavage by the at least one nucleozyme or the plurality of nucleozymes. An example of such a retrovirus associated disease is AIDS wherein the causative retrovirus is the human immunodeficiency virus (HIV).

- 21 -

The term "subject" is intended to include living organisms susceptible to retroviruses, e.g., mammals. Examples of subjects include humans, cats, and rats.

The language "therapeutically effective amount" is intended to include that amount capable of eliminating or significantly reducing the symptoms associated with retrovirus associated diseases. The amount may be determined on an individual basis and will be based, at least in part, on consideration of the severity of symptoms to be treated, the results sought and the size of the subject. Thus, a therapeutically effective amount may be determined by one of ordinary skill in the art employing such factors using no more than routine experimentation.

The nucleozymes of the present invention also may be used in conjunction with or in place of antisense RNA technology. That is, to control the expression of a gene by targeting an appropriate mRNA. A nucleozyme may be selected based on its ability to target a particular mRNA sequence and subsequently an effective amount of the nucleozyme may be administered to a subject. The effective amount would be that amount necessary to target the RNA and control expression of a selected gene.

The present invention also pertains to pharmaceutical compositions containing at least one nucleozyme and a pharmaceutically acceptable carrier. The language pharmaceutically acceptable carrier is intended to include carriers capable of being co-administered with the nucleozyme(s) while not adversely affecting the nucleozyme(s) catalytic

- 22 -

activity. The carrier may be solid or liquid or a gel. Examples of liquid carriers include water, an aqueous solution of a non-toxic salt, e.g., sterile physiological saline solutions, or aqueous solutions containing organic solvents, e.g., ethanol. Also suitable are emulsions, such as oil-in-water. Solid carriers may include nutritive carriers, e.g., sucrose or gelatin, or non-nutritive carriers, e.g., cellulose or talc.

The Preferred Embodiment

The preferred nucleozymes of the present invention are modeled after the "hammerhead" ribozyme. The moderate size of the catalytic hammerhead domains of RNA lends itself to chemical synthesis. As shown in FIG. 1, the conserved hammerhead domain of around fifty ribonucleotides found in naturally occurring RNA has been partitioned between a thirty-five unit catalytic fragment 10, the ribozyme, and a fourteen nucleotide substrate unit 12.

The all-DNA analog of the hammerhead domain is inactive in catalysis. It was determined that nucleozymes containing as few as four ribonucleotides out of a total of thirty five nucleotides have catalytic activity. Active hammerhead nucleozymes require the presence of ribonucleotides in particular at four positions, particularly the G9, G12, A13, and A29 positions shown in FIG. 2.

FIG. 2 is a table showing the chimeric polymers prepared along with the positions of ribonucleotides within the polymers. Nucleozymes are abbreviated as follows: DR and MR designate nucleozymes composed of

- 23 -

predominantly deoxyribonucleotides and 2'-methoxynucleotides, respectively. The following number indicates the number of ribonucleotides and the final number refers to a particular combination of ribonucleotides.

The all DNA analog of the hammerhead domain is inactive in catalysis. Several RNA/DNA mixed polymers appear as set forth and designated in the table of FIG 2. As shown in FIGs 3 and 4, the mixed polymer having ribonucleotides at positions 9, 10, 12, 13, 28, 29 and 30 of the ribozyme showed good catalytic activity (FIG 3). The nucleozyme containing the fewest ribonucleotides, DR4R3 and DR4R2, showed at least some activity. In FIGs 3 and 4, S indicates the mobility of the intact substrate and P, the product. RNA is the all-RNA ribozyme and nucleozymes are abbreviated as in FIG 1. The presence or absence of the enzymatic fragment is indicated by + or -, respectively in FIG 3. Lane 1 is a control lacking the added catalytic fragment in FIG 3. Lane 1 in FIG 4 is the 4.5 hour incubation of the substrate with neither Mg^{+2} nor nucleozyme and lane 2 is an incubation control in the presence of Mg^{+2} but no added nucleozyme. The samples were analyzed on 15% PAGE in 7M urea.

FIG 3 is a photograph showing the cleavage of radioactive substrates by OMe-nucleozymes. The S indicates the mobility of the intact substrate and P, the product. The reactions were performed as in FIG 2 except that the incubations of the substrate were with the MR5R nucleozyme, for four hours and the (Mg^{+2}) was varied. Lane 1 contained no Mg^{+2} ,

- 24 -

lane 2-10 mM, lane 3-20 mM, lane 4-30 mM and lane 5-50 mM. The OMe-nucleozymes are nucleozymes having synthons which are methoxysubstituted in the 2' position. The nucleozymes designated MR contain all methoxysubstituted nucleozymes rather than deoxyribonucleotides.

The catalytic activity of the nucleozymes was further evaluated by determining parameters including K_M and K_{cat} for some of the kinetic reactions. In addition, the K_{Mg} , K_S and k_3 of nucleozyme reactions were obtained to evaluate how extensive deoxyribonucleotide substitution effects Mg^{+2} cofactor binding. A summary of these results is presented in Table 1 below.

TABLE I Kinetic parameters of nucleozyme-catalyzed reactions^a

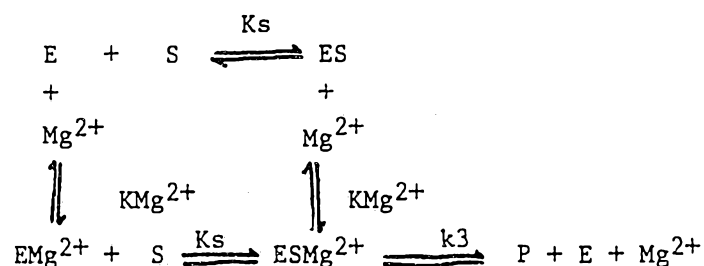
Substrate/ Nucleozyme	K_M (μM)	K_{cat} (1/min)	K_S (μM)	K_3 (1/min)	K_{Mg} (mM)
RS/DR4R3	4.2	0.004	5	0.013	23
RS/DR5R2	4.9	0.013	4	0.040	24
RS/DR7R	4.5	0.067	5	0.21	24
RS/RR	0.7	1.2	0.8	1.8	8.1

^a The kinetic experiments were performed under the standard conditions of FIG 2 with following differences: the final concentrations were: nucleozyme 0.05 μM ; substrate from 0.5 μM to 5 μM ; Mg^{2+} from 5 to 50 mM and the reaction times from 30 min to 6 h at 30°C. Initial rates were determined from the first 10% of the reactions. The chronology of substrate and metal ion addition is

- 25 -

currently not known. The values for K_s , k_3 and K_{Mg} were, therefore, determined from the following random assembly model for ternary complex formation:

The generalized reaction scheme used to determine the K values is depicted below.



The reaction scheme starts with the catalytic species, the substrate and the Mg^{+2} cofactor, traversing a ternary complex and yielding a product. The apparent K_m 's and K_s 's of the three nucleozymes are virtually identical and approximately five times those of the all-RNA ribozyme acting on an RNA substrate.

The chimeric polymers were synthesized as follows:

Ribonucleotide, Deoxyribonucleotide, or 2'-Methoxy Analogue Phosphoramidite Synthesis and Purification

Dry N-Acyl-5'-O-DMT-2'-O-sily-ribonucleoside (10 mmol, 1 eq) was dissolved in 30 ml dry THF in a 300 ml round bottom flask. 2, 4, 6-Collidine (75 mmol, 7.5 eq) was added followed by N-methylimidazole (5 mmol, 0.5 eq). (N,N-dispropylamino) (cyanoethyl) phosphonamidic chloride (20 mmol, 2.0 eq [22 mmol,

- 26 -

2.2 eq in the case of guanosine or other nucleotide bases with reactive lactams]) was then added dropwise over 5 min at room temperature. The reaction was clear at the start but a white precipitate formed within 3 min (this is the collidine hydrochloride salt). The reaction was complete after 1-2 hour (determined by TLC). The reaction was then placed in an ice bath and diluted with 100 ml (1 volume) ethyl acetate. 150 ml 5% NaHCO_3 was added slowly (the first 10 ml over 5 minutes). (In the case of guanosine the reaction was first quenched with 5 ml of absolute ethanol). Heat was generated by the quenching of excess phosphitylating reagent. The mixture was then transferred to a separatory funnel and a second volume of ethyl acetate (100 ml) was added after first rinsing out the reaction flask. The aqueous phase was removed and the organic phase washed with saturated NaCl. The combined aqueous washes were back extracted with 50 ml. ethyl acetate and the combined organic phases were dried over Na_2SO_4 . The solvent was removed in vacuo yielding a viscous oil. Coevaporation (x 5) with 50 ml toluene afforded the crude phosphoramidite as an offwhite foam or oil. Excess phosphinic acid and collidine caused it to be oily. After leaving the amidite under high vacuum overnight, resuspension and rotovaping of the amidite with methylene chloride and ethyl acetate usually produced a foam. The phosphoramidites were further purified by silica gel chromatography yielding a white foam in 75-85%

- 27 -

yields. This procedure was repeated for the preparation of the other desired phosphoramidites changing the initial protected nucleotide unit. The phosphoramidites were used to synthesize the mixed polymers using automated synthesis techniques commonly used for the synthesis of deoxyribonucleotides.

Automated Synthesis of Mixed Polymers

All syntheses were conducted on either a Gene Assembler Plus (Pharmacia), or a Cyclone (Milligen/Bioscience) synthesizer using standard protocols with an extended 12 min coupling step. A 30 fold excess (150 μ l of 0.1 M = 15 mg, ~ 15 μ mol) of the phosphoramidites and a 400 fold excess of tetrazole (400 μ l of 0.5M = 200 μ mol) relative to CPG bound 5'-hydroxyl was used in each coupling cycle. Synthesis scale was 0.5 μ mol. Average coupling yields on the Gene Assembler Plus, monitored by an on-line colorimeter, were ~99.0% and on the Cyclone 97-98%, determined by colorimetric quantitation of the trityl fractions. Reaction columns for 0.5 μ mol syntheses were Milligen/Bioscience 1.0 μ mol columns. Oligonucleotide synthesis reagents: 1) for GA plus: detritylation solution was 2% TCA in ethylene dichloride; capping was performed with 20% N-Methylimidazole in THF and 10% acetic anhydride/10% 2,6-lutidine in THF; oxidation solution was 0.02 MnO_2 , 1% lutidine, 10% water in THF. Baker

- 28 -

Bio-Analyzed grade acetonitrile was further dried over activated 4 A molecular sieves. Tetrazole solution (0.5 M in acetonitrile was obtained from Applied Biosystems. 2) for Cyclone: all standard DNA synthesis ancillary reagents were used.

Deprotection of Mixed Polymers

The CPG-bound mixed polymer was transferred from the synthesis column to a 4 ml glass screw top vial. 1 ml of ethanolic ammonia was added and heated at 55°C for 16 hr. After cooling to -20°C, the ethanolic ammonia was removed from the CPG beads and the CPG was washed with 0.5 ml of 50:50/ethanol:water which was then added to the ethanolic ammonia. The combined supernatants containing the oligoribonucleotide were dried to a white powder. To remove the silyl protecting groups, the ammonia-deprotected mixed polymer was resuspended in 50 µl of 50:50/ethanol:water and 600 µl of 1M TBAF/THF and left at room temperature for about 24 hr. The solution was then added directly to 10 ml of 0.1M TEAB and loaded onto a Qiagen 500 anion exchange cartridge (Qiagen Inc., Studio City, CA) prewashed with 10 µl of 0.05M TEAB, the nucleozyme was eluted with 7 ml of 2M TEAB and dried down to a white powder.

Gel Purification of Fully Deprotected Mixed Polymers

The oligomers were first checked by analytical PAGE (0.75 mm x 20 cm x 45 cm). 1 ODU of oligonucleotide in 5 µl H₂O was added to 5 µl

WO 93/15187

PCT/US93/00833

- 29 -

of deionized formamide and the total 10 μ l solution was loaded into a 1 cm wide lane. Following electrophoresis the gels were photographed by placing the gel over a fluorescent TLC plate and illuminating the gel with a UV lamp. The desired sequence was established according to electrophoretic mobility and purified by preparative electrophoresis using 1.5 mm thick gels with a single 12 cm wide lane. After electrophoresis the desired band was excised, crushed, placed into a sterile 5 ml test tube, and covered with 50 mM NH_4OAc pH 7.0. The tube was covered and kept at 37°C O/N. The supernatant was then removed and the gel pieces washed with an additional 1 ml of the extraction buffer. The combined washings were filtered through a 0.45 micron filter and loaded onto a 1 gram size Sep-Pak C18 cartridge (Waters-Millipore) prewashed with 5 ml each of acetonitrile, 50% acetonitrile/0.1M TEAB and 0.1M TEAB. After washing the cartridge with 5 ml of 0.1 M TEAB, the RNA was eluted in 5 ml 35:35:30 acetonitrile/methanol/water and dried down to a white powder.

The mixed polymers set forth in the Table of FIG 2 were prepared as described above.

AMENDED SHEET

The claims defining the invention are as follows:

1. An enzymatic nucleic acid molecule having catalytic activity comprising a plurality of catalytically critical sites and a plurality of catalytically non-critical sites the enzymatic nucleic acid molecule including a catalytic centre comprising the catalytically critical sites, and a plurality of catalytically non-critical sites, wherein the catalytically critical sites are ribonucleotides or nucleic acid analogues capable of participating in the catalytic activity of the enzymatic nucleic acid molecule and a majority of the catalytically non-critical sites in the catalytic centre are deoxyribonucleotides, the enzymatic nucleic acid molecule having an increased resistance to degradation compared to an all RNA counterpart.

2. An enzymatic nucleic acid molecule according to claim 1 wherein at least one of the sites is a nucleic acid analogue.

3. An enzymatic nucleic acid molecule having catalytic activity comprising a plurality of catalytically critical sites and a plurality of catalytically non-critical sites wherein the catalytically critical sites are ribonucleotides or nucleic acid analogues capable of participating in the catalytic activity of the enzymatic nucleic acid molecule; the catalytically non-critical sites are ribonucleotides, deoxyribonucleotides or nucleic acid analogues capable of being a monomer in a polymer chain which is capable of hybridising with DNA or RNA and wherein at least one of the sites is a nucleic acid analogue and at least one of the catalytically non-critical sites is a deoxyribonucleotide, the enzymatic nucleic acid molecule having an increased resistance to degradation compared to an all RNA counterpart.

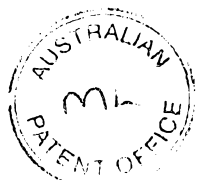
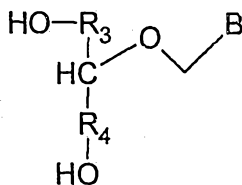
4. An enzymatic nucleic acid molecule according to any one of claims 1 to 3 wherein at least 50%, preferably at least 75%, more preferably at least 85%, and more preferably at least 90% of the catalytically non-critical sites are deoxyribonucleotides or nucleic acid analogues.

5. An enzymatic nucleic acid molecule according to any one of claims 1 to 3 wherein all of the catalytically non-critical sites are deoxyribonucleotides or nucleic acid analogues.

6. An enzymatic nucleic acid molecule according to claim 5 wherein all of the catalytically non-critical sites are deoxyribonucleotides.

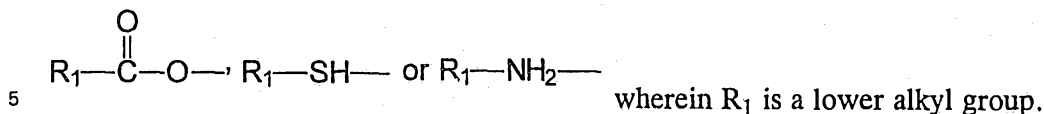
7. An enzymatic nucleic acid molecule according to any one of claims 2 to 5 wherein the nucleic acid analogues is acyclic.

8. An enzymatic nucleic acid molecule according to claim 7 wherein the nucleic acid analogues has the following formula:



wherein B is a base and R₃ and R₄ are each independently lower alkyl groups.

9. An enzymatic nucleic acid molecule according to claim 8 wherein B is selected from the group of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazolyl.

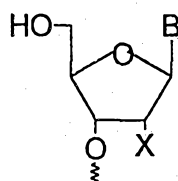


10. An enzymatic nucleic acid molecule according to any one of claims 2 to 6 wherein the nucleic acid analogue contains a heterocyclic ring.

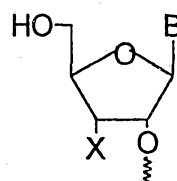
11. An enzymatic nucleic acid molecule according to claim 10 wherein the nucleic acid analogue has a formula selected from the group consisting of:

10

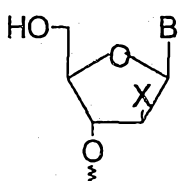
Formula (I)



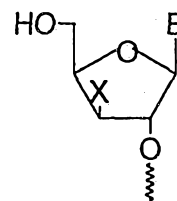
Formula (III)



Formula (II)



Formula (IV)



15

wherein B is a base and X is a coordinating ligand for a divalent metal ion.

12. An enzymatic nucleic acid molecule according to claim 11 wherein B is selected from the group consisting of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazolyl; and X is selected from the group consisting of -OR₁, F, -R₂OH, and -CH₂F, wherein R₁ is a lower alkyl group and R₂ is a lower alkyl chain.

13. An enzymatic nucleic acid molecule according to claim 12 wherein the nucleic acid analogue has a formula as shown in (I) and X is methoxy.

14. An enzymatic nucleic acid molecule having catalytic activity comprising a plurality of catalytically critical sites and a plurality of catalytically non-critical sites wherein the catalytically critical sites are ribonucleotides or nucleic acid analogues capable of participating in the catalytic activity of the enzymatic nucleic acid molecule; the catalytically non-critical sites are ribonucleotides, deoxyribonucleotides or nucleic acid analogues capable of being subunits in a chain molecule which is capable of hybridising with DNA or RNA and wherein at least one of the sites is a nucleic acid analogue other than a 2'-fluoro, 2'-amino or 2'-alkoxy nucleotide analogue, the enzymatic nucleic acid



molecule having an increased resistance to degradation compared to an all ribonucleotide counterpart.

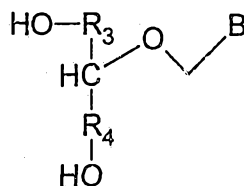
15. An enzymatic nucleic acid molecule according to claim 14 wherein at least 50%, preferably at least 75%, more preferably at least 85% and most preferably at least 90% of the catalytically non-critical sites are deoxyribonucleotides or nucleic acid analogues.

16. An enzymatic nucleic acid molecule according to claim 15 wherein all of the catalytically non-critical sites are deoxyribonucleotides or nucleic acid analogues.

17. An enzymatic nucleic acid molecule according to claim 16 wherein all of the catalytically non-critical sites are deoxyribonucleotides.

18. An enzymatic nucleic acid molecule according to any one of claims 14 to 17 wherein the nucleic acid analogue is acyclic.

19. An enzymatic nucleic acid molecule according to claim 18 wherein the nucleic acid analogue has the following formula:



wherein B is a base and R₃ and R₄ are each independently lower alkyl groups.

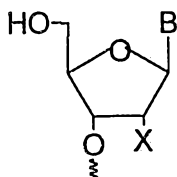
20. An enzymatic nucleic acid molecule according to claim 19 wherein B is selected from the group of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazolyl.

20 $\text{R}_1-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-\text{R}_1-\text{SH}-$ or R_1-NH_2- wherein R₁ is a lower alkyl group.

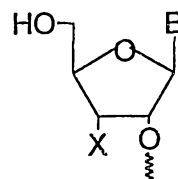
21. An enzymatic nucleic acid molecule according to any one of claims 14 to 17 wherein the nucleic acid analogue contains a heterocyclic ring.

22. An enzymatic nucleic acid molecule according to claim 21 comprising at least one nucleotide analogue having a formula selected from the group consisting of:

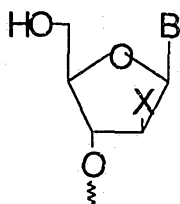
25 Formula (I)



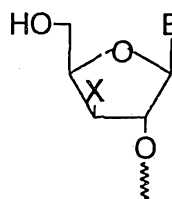
Formula (III)



Formula (II)



Formula (IV)



wherein B is a base and X is a coordinating ligand for a divalent metal ion.

23. An enzymatic nucleic acid molecule according to claim 22 wherein B is selected from the group consisting of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazolyl; and X is selected from the group consisting of $-OR_1$, F, $-R_2OH$, and $-CH_2F$, wherein R_1 is a lower alkyl group and R_2 is a lower alkyl chain except when the nucleic acid analogue has a formula according to Formula (I) when X is selected from the group consisting of $-OR_1$, or $-R_2OH$, wherein R_1 is a lower alkyl group and R_2 is a lower alkyl chain.

24. An enzymatic nucleic acid molecule according to claim 23 wherein the nucleic acid analogue has a formula as shown in (I) and X is methoxy.

25. An enzymatic nucleic acid molecule according to any one of the preceding claims wherein, the enzymatic nucleic acid molecule has less than about 100 total sites, preferably less than 80 sites, more preferably less than 70 sites, still more preferably less than 50 sites, and most preferably less than 35 sites.

26. An enzymatic nucleic acid molecule according to any one of the preceding claims wherein the enzymatic nucleic acid molecule has a sequence corresponding to a modified ribozyme selected from the group consisting of hammerhead and hairpin ribozymes.

27. An enzymatic nucleic acid molecule according to claim 26 wherein the ribozyme has a sequence corresponding to the hammerhead ribozyme.

28. An enzymatic nucleic acid molecule according to claim 27 wherein the catalytically critical sites are at the positions corresponding to the nucleotides G9, G12, A13 and A29 of the hammerhead ribozyme as shown in fig 1.

29. An enzymatic nucleic acid molecule according to any one of the preceding claim wherein all the catalytically critical sites are ribonucleotides.

30. An enzymatic nucleic acid molecule according to any one of the preceding claims wherein the enzymatic nucleic acid molecule further comprises a non-nucleotide spacer molecule.

31. An enzymatic nucleic acid molecule according to any one of the preceding claims wherein the increased resistance is resistance to enzymatic or chemical degradation.



32. An enzymatic nucleic acid molecule according to claim 31 wherein the resistance is to enzymatic degradation and the enzymatic nucleic acid molecule is at least 80%, preferably at least 90%, intact at an RNase A log concentration of -2.5.

33. An enzymatic nucleic acid molecule according to claim 31 wherein the
5 resistance is to enzymatic degradation and the enzymatic nucleic acid molecule is at least 75%, preferably at least 90%, intact at an RNase A log concentration of -1.5.

34. A method of making a enzymatic nucleic acid molecule according to any one of claims 1 to 33 comprising:

phosphitylating protected RNA, deoxyribonucleotides or nucleic acid analogues
10 under conditions to form substantially pure-protected phosphoramidites of a single isomer;
coupling the protected phosphoramidites together forming a protected chimeric polymer chain; and

deprotecting the chimeric polymer chain under conditions which completely deprotect the chimeric polymer.

15 35. The method as claimed in claim 34 wherein the phosphitylating agent in the phosphitylating step is (N,N-diisopropylamino) (cyanoethyl) phosphonamidic chloride.

36. A method as claimed in claim 34 or claim 35 wherein the phosphitylating step is conducted in the presence of 2,4, 6-collidine and N-methylimidazole.

37. A method as claimed in claim 35 or 36 wherein the deprotecting step is
20 conducted in the presence of ethanolic ammonia.

38. An enzymatic nucleic acid molecule according to any one of claims 1 to 33 which is free of undesired isomeric products.

39. An enzymatic nucleic acid molecule according to any one of claims 1 to 33 which is free of isomeric products containing 5'-2' linkages.

25 40. A method for selectively cleaving an RNA substrate, comprising:
contacting the substrate with a enzymatic nucleic acid molecule according to any one of claims 1 to 33 which targets a specific sequence in the substrate for cleavage.

41. A method according to claim 40 wherein the RNA substrate is a messenger RNA.

30 42. A pharmaceutical composition comprising:
at least one enzymatic nucleic acid molecule according to any one of claims 1 to 33; and a pharmaceutically acceptable carrier.

43. The use of a enzymatic nucleic acid molecule according to any one of claims 1 to 33 in the manufacture of a medicament for the treatment of a retrovirus associated
35 disease.

44. The use of enzymatic nucleic acid molecule according to claim 43 wherein a plurality of enzymatic nucleic acid molecules according to claims 1 to 33 are used.

45 45. The use of a enzymatic nucleic acid molecule according to claim 43 or claim 45 wherein the retrovirus associated disease is associated with the human immunodeficiency virus.



46. A method of polymerising an oligonucleotide molecule comprising:

contacting a template with a population of nucleotide monomer units and a enzymatic nucleic acid molecule according to any one of claims 1 to 33 such that an oligonucleotide complementary to the template is formed.

5 47. A method as claimed in claim 46 wherein the oligonucleotide formed is an oligoribonucleotide.

48. A method as claimed in claim 46 wherein the oligonucleotide formed is an oligodeoxyribonucleotide.

49. The use of a enzymatic nucleic acid molecule according to any one of claims 1
10 to 33 in the manufacture of a medicament for controlling the expression of a gene.

50. An enzymatic nucleic acid molecule, comprising a plurality of catalytically critical sites comprising a catalytic centre and a plurality of catalytically non-critical sites, wherein a majority of the sites in said catalytic centre are nucleic acid analogues or deoxyribonucleotides; and

15 wherein a majority of the sites in said enzymatic nucleic acid molecule are nucleic acid analogues or deoxyribonucleotides, and

wherein at least one of said nucleic acid analogues or deoxyribonucleotides, of said majority of the sites in said enzymatic nucleic acid molecule, comprises, at the 2'-position of a sugar moiety, an analogue group different from 2'-alkoxy or 2'-OH.

20 51. The enzymatic nucleic acid molecule of claim 50, further comprising at least one site a nucleic acid analogue comprising a 2'-methoxy analogue group.

52. The enzymatic nucleic acid molecule of claim 51, further comprising at least one site, a nucleotide analogue which does not comprise a purine or pyrimidine base.

53. The enzymatic nucleic acid molecule of claim 51, further comprising at least
25 one site, a nucleotide analogue comprising a 2'-amino analogue group.

54. An enzymatic nucleic acid molecule, comprising a plurality of catalytically critical sites and a plurality of catalytically non-critical sites, wherein a plurality of the catalytically critical sites are ribonucleotides; and

wherein a plurality of the sugar moieties in said enzymatic nucleic acid molecule
30 comprise, at their 2'-positions, analogue groups other than hydroxyl alone,

wherein at least two said analogue groups other than hydroxyl alone are different,
and

wherein at least one said analogue group other than hydroxyl alone is different from 2'-halo and does not comprise a nitrogen atom linked to the 2'-carbon atom.

35 55. The enzymatic nucleic acid molecule of claim 54, wherein at least one of said sugar moieties comprises, at its 2'-position, a 2'-methoxy analogue group.

56. The enzymatic nucleic acid molecule of claim 54, having a hairpin motif.

57. The enzymatic nucleic acid molecule of claim 54, having a hammerhead motif.

58. The enzymatic nucleic acid molecule of claim 57, having a formula 3'-V-W-

40 X-Y-Z-5';



wherein V and Z are sequences which can base pair with a separate nucleic acid molecule and X is a sequence which can base pair *inter se* to form a stem-loop structure;

wherein W comprises 3'-A^bAAG-5', Y comprises two 3'-AG^b-5'; wherein b represents a nucleotide having a 2'-OH group.

5 59. The enzymatic nucleic acid molecule of claim 58, wherein at least two said analogue groups other than hydroxyl alone are selected from the group consisting of 2'-O-methyl, 2'-amino and 2'-fluoro.

60. The enzymatic nucleic acid molecule of claim 59, wherein at least one said analogue group at said 2'-position is 2'-O-methyl.

10 61. The enzymatic nucleic acid molecule of claim 59, wherein at least one said analogue group at said 2'-position is 2'-amino.

62. An enzymatic nucleic acid molecule having a hammerhead motif, wherein said molecule has the formula 3'-V-W-X-Y-Z-5',

wherein V and Z are sequences which can base pair with a separate nucleic acid molecule and X is a sequence which can base pair *inter se* to form a stem-loop structure;

wherein W comprises 3'-A^bAAG-5', Y comprises two 3'-AG^b-5'; wherein b represents a nucleotide having a 2'-OH group;

and wherein at least two of the sites of said enzymatic nucleic acid molecule 20 comprise a deoxyribonucleotide and a nucleic acid analogue, or two different nucleic acid analogues, wherein the difference does not consist solely of different selections of the bases adenine, guanine, cytosine, uracil, or thymine.

63. The enzymatic nucleic acid molecule of claim 62, wherein at least two of the sugar moieties in said enzymatic nucleic acid molecule comprise, at their 2'-positions, 25 analogue groups other than hydroxyl alone,

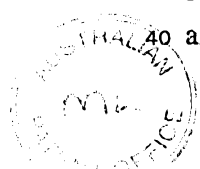
wherein at least two said analogue groups other than hydroxyl alone are different.

64. The enzymatic nucleic acid molecule of claim 62, comprising at least one nucleic acid analogue, wherein said nucleic acid analogue comprises, at the 2'-position of a sugar moiety, a 2'-alkoxy analogue group.

30 65. An enzymatic nucleic acid molecule, comprising at least one nitrogen-containing backbone linkage.

66. An enzymatic nucleic acid molecule having enzymatic activity to cleave a separate ribonucleic acid molecule, comprising

a nucleotide base-containing molecule having a hammerhead motif which has the 35 formula 3'-V-W-X-Y-Z-5', wherein V and Z are sequences which can base pair with said separate ribonucleic acid molecule and X is a sequence which can base pair *inter se* to form a hairpin; and wherein W comprises 3'-A^bAAG-5', Y comprises two 3'-AG^b-5'; wherein b represents a nucleotide having a 2'-OH group; and wherein at least one of the sugar moieties in said enzymatic nucleic acid molecule comprises, at its 2'-position, an analogue group other than hydroxyl alone, wherein said analogue group is not 2'-alkoxy



or 2'-halo, and does not comprise a 2'-O-containing derivative or a nitrogen atom linked to the 2'-C, wherein said analogue group at each 2'-position may be the same or different.

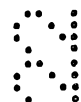
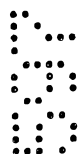
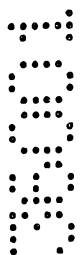
67. The enzymatic nucleic acid molecule of claim 66, further comprising at least one phosphorothioate linkage.

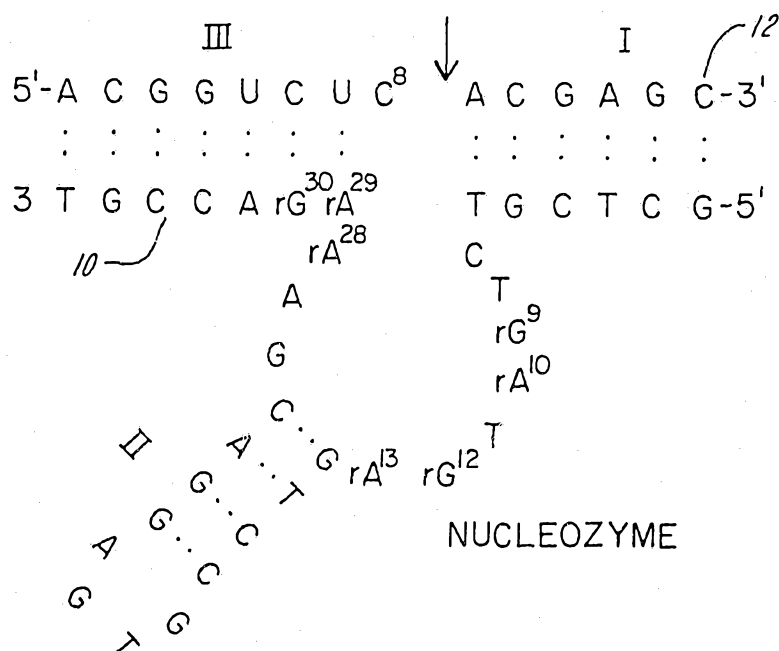
5 68. The enzymatic nucleic acid molecule of claim 67, further comprising at least one nucleotide analogue which does not comprise a purine or pyrimidine base.

Dated 20 May, 1997
Massachusetts Institute of Technology

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

10



**FIG. 1**

	POSITION OF RIBONUCLEOTIDE		
	rG ⁹ rA ¹⁰	rG ¹² rA ¹³	rA ²⁸ rA ²⁹ rG ³⁰
NUCLEOZYME			
DR2R	rG	rA	--
DR3R	rG	rA	rA
DR4R1	rG	rA	rArG
DR4R2	rG	rGrA	rG
DR4R3	rG	rGrA	rA
DR5R2	rG	rGrA	rArG
DR5R3	rGrA	rGrA	rG
DR6R2	rGrA	rGrA	rArG
DR7R	rGr	GrA	rArArG
OMe-NUCLEOZYME			
MR2R	rG	rA	--
MR3R	rG	rA	rA
MR5R	rG	rGrA	rArG

FIG. 2
SUBSTITUTE SHEET

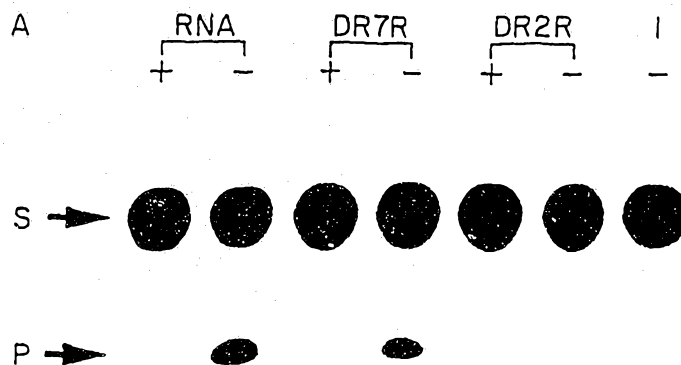


FIG. 3

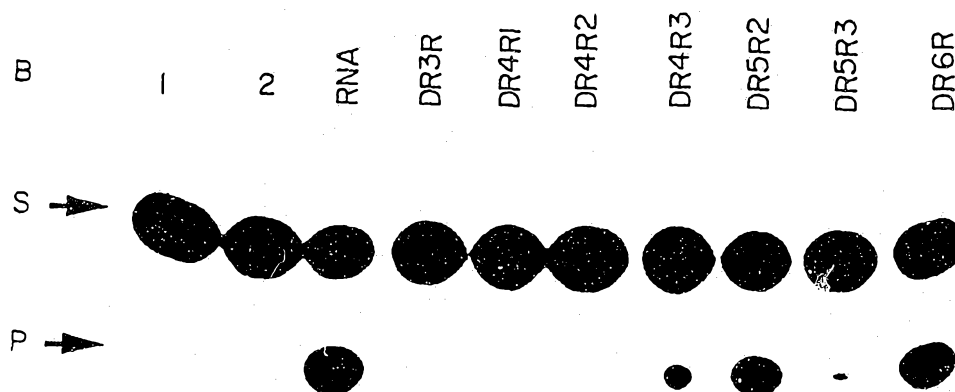


FIG. 4

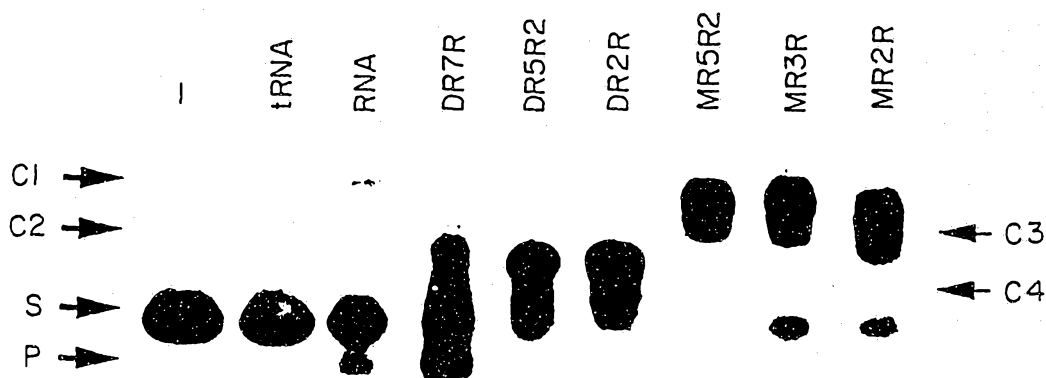
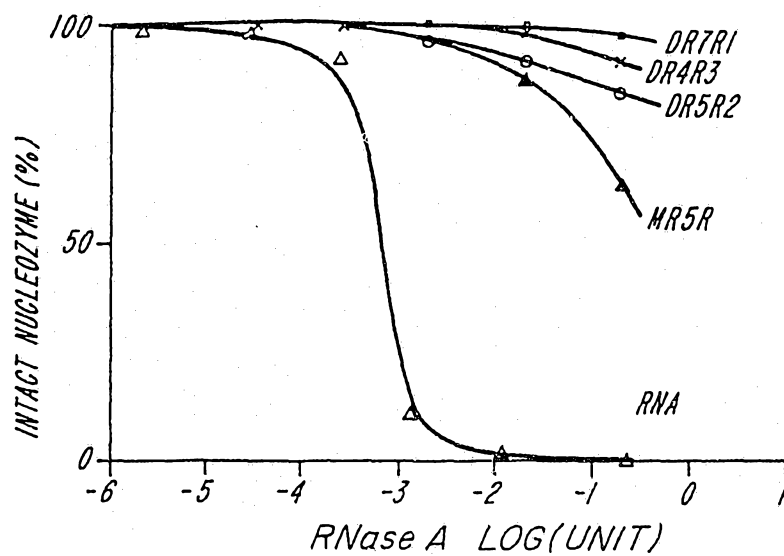
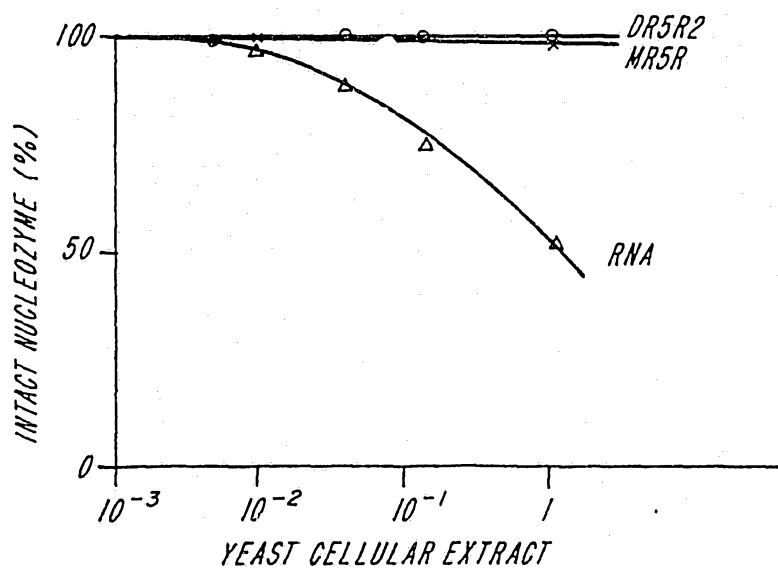


FIG. 5
SUBSTITUTE SHEET

**FIG. 6****FIG. 7**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/00833

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5	C12N9/00; A61K31/70;	C07H21/00; C12P19/34
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A61K ; C07H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 103 162 (CITY OF HOPE) 21 March 1991 see the whole document	1-3, 13-20 22-23, 25-36, 47-57, 63-66, 70-71 73-74, 78-79
Y	---	56-62, 67-69
	---	---/--
¹⁰ Special categories of cited documents : ^{"A"} document defining the general state of the art which is not considered to be of particular relevance ^{"E"} earlier document but published on or after the international filing date ^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) ^{"O"} document referring to an oral disclosure, use, exhibition or other means ^{"P"} document published prior to the international filing date but later than the priority date claimed ^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention ^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step ^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. ^{"&"} document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 27 MAY 1993		Date of Mailing of this International Search Report 03. 06. 93
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	NUCLEIC ACIDS RESEARCH vol. 18, no. 18, 25 September 1990, ARLINGTON, VIRGINIA US pages 5433 - 5441 SCARINGE, S.A. ET AL. 'Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl protected ribonucleoside phosphoramidites' cited in the application	56-62
Y	see the whole document	56-62, 67-69
X	SCIENCE vol. 253, 19 July 1991, LANCASTER, PA US pages 314 - 317 PIEKEN, W.A. ET AL. 'Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes' see the whole document	1,4-6, 10-11, 13-20, 22-23, 25-34, 37-39 42-43, 47-48, 52,54, 56,63,70
X	BIOCHEMISTRY vol. 30, no. 16, 1991, EASTON, PA US pages 4020 - 4025 PERREAULT, J.-P. ET AL. 'Relationship between 2'-hydroxyls and magnesium binding in the hammerhead RNA domain: a model for ribozyme catalysis' cited in the application see the whole document	1-3, 13-20, 34-35, 47, 52-54, 63-66,70
X	WO,A,8 804 300 (UNIVERSITY PATENTS, INC.) 16 June 1988 see page 16, line 10 - page 17, line 24; claim 72	75-76
X	SCIENCE vol. 251, 20 March 1991, LANCASTER, PA US pages 1605 - 1608 DOUDNA, J.A. ET AL. 'A multisubunit ribozyme that is a catalyst of and template for complementary strand RNA synthesis' see the whole document	75-76

-/--

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/00833

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 71-73 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOCHEMISTRY vol. 31, no. 21, 2 June 1992, EASTON, PA US pages 5005 - 5009 YANG, J.-H. ET AL. 'Minimum ribonucleotide requirement for catalysis by the RNA hammerhead domain' see the whole document ---	1,4-6, 10-34, 37-39, 42-52, 54,56, 58-63 67-70
P,X	EMBO JOURNAL vol. 11, no. 5, May 1992, EYNSHAM, OXFORD GB pages 1913 - 1919 PAOLELLA, G. ET AL. 'Nuclease resistant ribozymes with high catalytic activity' see the whole document ---	1,4-6, 10-20, 22-34, 37-39, 42-43, 45-52,63 70
P,X	WO,A,9 207 065 (MAX-PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 30 April 1992 see page 4, line 6 - page 7, line 3 see claims; example 4 -----	1,4-6, 10-11 13-20 22-34, 37-39, 42-44 47-52, 54,63, 70-71, 73-79

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9300833
SA 70071

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

27/05/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9103162	21-03-91	AU-A- 5817790	08-04-91
		DE-T- 4091533	30-01-92
		EP-A- 0451221	16-10-91
		EP-A- 0506666	07-10-92
		GB-A- 2242432	02-10-91
		JP-T- 4502256	23-04-92
		WO-A- 9206988	30-04-92
		US-A- 5149796	22-09-92
WO-A-8804300	16-06-88	US-A- 4987071	22-01-91
		EP-A- 0291533	23-11-88
		JP-T- 1501445	25-05-89
		US-A- 5037746	06-08-91
		US-A- 5116742	26-05-92
		US-A- 5093246	03-03-92
WO-A-9207065	30-04-92	None	