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(54) Title: METHOD AND REAGENT FOR INHIBITING HEPATITIS C VIRUS REPLICATION (57) Abstract An enzymatic RNA molecule which specifically cleaves RNA of a hepatitis C virus.		

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DESCRIPTIONMethod and Reagent for Inhibiting
Hepatitis C Virus ReplicationBackground of the Invention

This application is a continuation-in-part of Draper, entitled "Method and Reagent For Inhibiting Hepatitis C Virus Replication", filed May 14, 1992, assigned U.S. Serial No. 07/882,888, the whole of which is hereby
5 incorporated by reference herein.

This invention relates to reagents useful as inhibitors of hepatitis C virus (HCV) replication and gene expression.

10 The following is a discussion of relevant art, none of which is admitted to be prior art to the pending claims.

The Hepatitis C virus group is responsible for most cases of non-A, non-B hepatitis. Although non-A, non-B hepatitis may be caused by many different viruses, the
15 principal cause is HCV. The infection is transmitted through blood transfusions (25-50% of hemophiliacs have chronic hepatitis), percutaneous infusion from illicit drug usage (25-50% of all drug users have non-A, non-B hepatitis), renal organ transplant (where most late
20 mortality is due to non-A, non-B hepatitis), and maternal-neonatal transfer. Up to 20% of sporadic outbreaks of hepatitis are caused by HCV.

The association of hepatitis C virus in posttransfusion hepatitis (>95% of the cases) and the
25 subsequent observation that infection with this virus, more so than with hepatitis B virus, correlates strongly with the development of hepatocellular carcinoma and liver cirrhosis has led to intense study of the molecular biology of the viral genes.

30 Hepatitis C virus (HCV) is a positive-stranded, enveloped virus which is evolutionarily related to the flaviviruses and pestiviruses. The HCV genome consists of

a 5' untranslated region (5' UTR), a long open reading frame of between 9030 and 9099 nucleotides and a 3' UTR. The 3' end of the genome has been reported to contain a short stretch of poly(A) in type I strains, but this has not been confirmed in type II, III or IV strains. It has been reported that sequences in the 5' UTR exhibit negative translational control over viral gene expression, but the major regulation of HCV gene expression comes from the proteolytic cleavages of the polyprotein. Various proteolytic cleavages of the polyprotein are essential for the efficient expression of replication-associated viral proteins after translation. The resultant replicase proteins interact with a region of between 27 and 45 nucleotides in the 3' UTR which functions as a structural cue for the initiation of viral genomic replication.

Cleavage of the polyprotein of HCV uses both cellular and virally-encoded proteases and generates at least nine proteins. A tenth viral protein (a second protease) has been reported to be generated by alternative cleavage of the polyprotein, but has not been confirmed. Confirmed virally-encoded proteins can be classified as structural or non-structural proteins. The structural proteins of the virus are the C protein (22kD), E glycoprotein (35 kD) and the E2/NS1 glycoprotein (58 kD). The non-structural proteins include the NS2 (53 kD), NS3 (70 kD), NS4a (8 kD), NS4b (27 kD), NS5a (58 kD) and NS5b (68 kD).

The C protein is a hydrophobic protein which constitutes the core structural component of the viral particle and may play a role in localizing the viral particle to the cellular membrane. There is also evidence from transient assays that the core protein may function as a transactivator of gene expression. This protein is essential to viral replication and appears to be highly conserved at the nucleotide level of homology among known types of HCV.

The envelope proteins are necessary for viral replication but their nucleotide (and amino acid)

sequences diverge significantly between types of HCV. The NS3 protein functions as the major viral protease and mutations in this protein lead to inactivation of viral replication by inhibiting the processing of the polyprotein. The nucleotide sequence of the NS3 region is highly conserved among viral isolates.

The level of infidelity exhibited by the HCV RNA replicase has been indirectly quantified by assessing the sequential appearance of nucleotide substitutions in the genomes of HCV samples obtained from the same patient. These studies suggested that the nucleotide divergence was minimal but it may have been an underestimate of the actual infidelity of the replicase because the recovered samples were actually pre-selected by growth in the patient. Unacceptable substitutions would have been lost during the outgrowth of the virus.

The replication of HCV in hepatocytes is integrally intertwined with the replication of other hepatitis viruses. Although hepatitis delta virus (HDV) is quite distinct from HCV, the life cycles of the two viruses can be interdependent. Normally HDV depends upon the replication of Hepatitis B Virus (HBV) to supply capsid proteins and other functions necessary for the assembly of infectious HDV particles. Because HCV inhibits HBV and Hepatitis A virus (HAV) growth in co-infected cells, the replication of HCV can also inhibit the maturation of HDV. The use of HDV as a gene vector for treatment of HCV infections would not be influenced by this interference because gene expression of the HDV genome is apparently uninhibited by HCV.

Summary of the Invention

The invention features novel enzymatic RNA molecules, or ribozymes, and methods for their use for inhibiting HCV replication. Such ribozymes can be used in a method for treatment of diseases caused by these related viruses in man and other animals, including other primates.

Ribozymes are RNA molecules having an enzymatic activity which is able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence specific manner. Such enzymatic RNA molecules can be targeted to
5 virtually any RNA transcript, and efficient cleavage achieved in vitro. Kim et al., 84 Proc. Nat. Acad. of Sci. USA 8788, 1987, Hazeloff et al., 234 Nature 585, 1988, Cech, 260 JAMA 3030, 1988, and Jefferies et al., 17 Nucleic Acid Research 1371, 1989.

10 Ribozymes act by first binding to a target RNA. Such binding occurs through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA which acts to cleave the target RNA. Thus, the ribozyme first recognizes and then
15 binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After a ribozyme has bound and cleaved
20 its RNA target it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology
25 (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of
30 the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of
35 binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA

target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in
5 base pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

This class of chemical cleavers will exhibit a high degree of specificity for only the viral mRNA in infected
10 cells. A ribozyme molecule targeted to a highly conserved sequence region will allow the treatment of many strains of human HCV with a single compound. No treatment exists which specifically attacks expression of the viral gene(s) of HCV. Current treatment protocols (interferon and
15 ribavirin) consist of treating the symptoms of the disease, not the viral replication. Upon cessation of current treatment protocols, a rebound of disease ensues because the treatment does not impact the latency of the virus or actual viral replication. The cleavage of viral
20 RNA offers a novel treatment which will reduce virus replication and establishment of latency in newly infected cells.

The methods of this invention can be used to treat human hepatitis C virus infections, which include both
25 acute and chronic virus infection and HCV-induced hepatocyte transformation. The utility can be extended to other species of HCV which infect non-human animals, where such infections are of veterinary importance. Further, the utility may be extended to other members of the pestivirus
30 and flavivirus families.

Treatment will occur at the time of active viral infection and will reduce virus loads in the infected cells and also disable viral replication. Ribozyme treatment may also be used as a means of creating
35 defective genomes which can be used in vaccines.

Thus, in the first aspect the invention features an enzymatic RNA molecule (or ribozyme) which specifically cleaves HCV RNA.

Preferred cleavage sites are within regions required for viral replication, e.g., protein synthesis, such as the 5' non-translated region of the HCV genome. This region is believed to control the translation of viral proteins in a manner which is reminiscent of the cap independent translational control of picornaviruses. Disruption of this region in the RNA results in deficient protein synthesis as well as incomplete DNA synthesis (and inhibition of transcription from the defective genomes).

Alternative regions outside of the 5' non-translated region also make suitable targets of ribozyme-mediated inhibition of HCV replication. Such targets include the [mRNA] genomic regions which encode the viral structural protein, E2/NS1, and the regions encoding the nonstructural proteins, especially the NS3 protease and NS2/NS3 hybrid proteases. Selection of particular target regions will depend upon the secondary structure of the genome. To avoid the potential of viral escape from ribozyme therapy, by random base changes in the genomic RNA, targeting of multiple regions within the 5' UTR, or the C and NS3 genes is anticipated. Multiple targeting will also minimize the ability of the virus to utilize point mutations for escape from immune surveillance. The phase variation which accompanies amino acid substitutions in the surface proteins (and result from point mutations in the genome of HCV) has been proposed to account for the viral persistence which is typical of HCV infections. Thus, two or more different ribozymes can be used in this invention for therapeutic treatment.

By "gene" is meant to refer to either the protein coding regions of the cognate mRNA, or any regulatory regions in the RNA which regulate synthesis of the protein or stability of the mRNA.

By "enzymatic RNA molecule" it is meant an RNA molecule which has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave RNA in that target. That is, the enzymatic RNA molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA to allow the cleavage to occur. 100% complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. By "equivalent" RNA to HCV is meant to include those naturally occurring RNA molecules associated with viral caused diseases in various animals, including humans, and other primates. These viral RNAs have similar structures and equivalent genes to each other.

In preferred embodiments, the enzymatic RNA molecule is formed in a hammerhead motif, but may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNaseP RNA (in association with an RNA guide sequence). Examples of such hammerhead motifs are described by Rossi et al., 8 AIDS RESEARCH AND HUMAN RETROVIRUSES 183, 1992, of hairpin motifs by Hampel et al., RNA CATALYST FOR CLEAVING SPECIFIC RNA SEQUENCES, filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100 filed September 20, 1988, Hampel and Tritz, 28 Biochemistry 4929, 1989 and Hampel et al., 18 Nucleic Acids Research 299, 1990, and an example of the hepatitis delta virus motif is described by Perrotta and Been, 31 Biochemistry 16, 1992, of the RNaseP motif by Guerrier-Takada et al., 35 Cell 849, 1983, and of the group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic RNA molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene

RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

In particularly preferred embodiments, the RNA which is cleaved by the ribozyme is in the 5' UTR of HCV genomic RNA or in the C protein or NS3 ORFs. Nucleotide numbers in the HCV genome are given to delineate the 5' ends of the target regions. Primary sequence data is taken from Tanaka et al, 23 Virus Res. 39, 1992. For comparison, the HCV sequence data of Choo et al 88 Proc. Natl. Acad. Sci. USA 2451 1991 was used.

TABLE 1. HCV mRNA target sequences.

	Nucleotide No.	Target Sequence ^a	Seq. ID. NO.
15	27	CGACACU C CACCAUA	Seq. ID. NO. 001
	114	GCAGCCU C CAGGACC	Seq. ID. NO. 002
	128	CCCCCU C CCGGGAG	Seq. ID. NO. 003
	148	UAGUGGU C UGCGGAA	Seq. ID. NO. 004
	165	GGUGAGU A CACCGGA	Seq. ID. NO. 005
20	175	CCGGAAU U GCCAGGA	Seq. ID. NO. 006
	199	CCUUUCU U GGAUCAA	Seq. ID. NO. 007
	213	ACCCGCU C AAUGCCU	Seq. ID. NO. 008
	252	GACUGCU A GCCGAGU	Seq. ID. NO. 009
	260	GCCGAGU A GUGUUGG	Seq. ID. NO. 010
25	265	GUAGUGU U GGGUCGC	Seq. ID. NO. 011
	270	GUUGGGU C GCGAAAG	Seq. ID. NO. 012
	288	UUGUGGU A CUGCCUG	Seq. ID. NO. 013
	298	GCCUGAU A GGGUGCU	Seq. ID. NO. 014
	306	GGGUGCU U GCGAGUG	Seq. ID. NO. 015

	325	GGGAGGU C UCGUAGA	Seq. ID. NO. 016
	327	GAGGUCU C GUAGACC	Seq. ID. NO. 017
	330	GUCUCGU A GACCGUG	Seq. ID. NO. 018
	407	AGGACGU C AAGUUCC	Seq. ID. NO. 019
5	412	GUCAAGU U CCCGGGC	Seq. ID. NO. 020
	413	UCAAGUU C CCGGGCG	Seq. ID. NO. 021
	426	CGGUGGU C AGAUCGU	Seq. ID. NO. 022
	472	CCCACGU U GGGUGUG	Seq. ID. NO. 023
	489	CGCGACU A GGAAGAC	Seq. ID. NO. 024
10	498	GAAGACU U CCGAACG	Seq. ID. NO. 025
	499	AAGACUU C CGAACGG	Seq. ID. NO. 026
	508	GAACGGU C GCAACCU	Seq. ID. NO. 027
	534	ACAACCU A UCCCCAA	Seq. ID. NO. 028
	536	AACCUAU C CCCAAGG	Seq. ID. NO. 029
15	546	CAAGGCU C GCCGACC	Seq. ID. NO. 030
	561	CGAGGGU A GGGCCUG	Seq. ID. NO. 031
	573	CUGGGCU C AGCCUGG	Seq. ID. NO. 032
	583	CCUGGGU A CCCUUGG	Seq. ID. NO. 033
	588	GUACCCU U GGCCCCU	Seq. ID. NO. 034
20	596	GGCCCCU C UAUGGCA	Seq. ID. NO. 035
	598	CCCCUCU A UGGCAAU	Seq. ID. NO. 036
	632	GAUGGCU C CUGUCAC	Seq. ID. NO. 037
	637	CUCCUGU C ACCCCGC	Seq. ID. NO. 038
	649	CGCGGCU C CCGGCCU	Seq. ID. NO. 039
25	657	CCGGCCU A GUUGGGG	Seq. ID. NO. 040
	660	GCCUAGU U GGGGCC	Seq. ID. NO. 041

	696	GCGCAAU C UGGGUAA	Seq. ID. NO. 042
	707	GUAAGGU C AUCGAUA	Seq. ID. NO. 043
	710	AGGUCAU C GAUACCC	Seq. ID. NO. 044
	714	CAUCGAU A CCCUCAC	Seq. ID. NO. 045
5	730	UGC GGCU U CGCCGAC	Seq. ID. NO. 046
	731	GCGGCUU C GCCGACC	Seq. ID. NO. 047
	748	AUGGGGU A CAUCCG	Seq. ID. NO. 048
	752	GGUACAU U CCGCUCG	Seq. ID. NO. 049
	753	GUACAUU C CGCUCGU	Seq. ID. NO. 050
10	758	UUCCGCU C GUCGGCG	Seq. ID. NO. 051
	761	CGCUCGU C GGCGCCC	Seq. ID. NO. 052
	773	CCCCCU A GGGGGCG	Seq. ID. NO. 053
	806	AUGGUGU C CGGGUUC	Seq. ID. NO. 054
	812	UCCGGGU U CUGGAGG	Seq. ID. NO. 055
15	813	CCGGGUU C UGGAGGA	Seq. ID. NO. 056
	832	GUGAACU A CGCAACA	Seq. ID. NO. 057
	847	GGGAACU U GCCCGGU	Seq. ID. NO. 058
	855	GCCCGGU U GCUCUUU	Seq. ID. NO. 059
	859	GGUUGCU C UUUCUCU	Seq. ID. NO. 060
20	982	AUUGUGU A UGAGGCA	Seq. ID. NO. 061
	1001	GCAUGAU C AUGCAUA	Seq. ID. NO. 062
	1022	GGUGCGU A CCCUGCG	Seq. ID. NO. 063
	1031	CCUGCGU U CGGGAGA	Seq. ID. NO. 064
	1032	CUGCGUU C GGGAGAA	Seq. ID. NO. 065
25	1048	AACGCCU C CCGUUGU	Seq. ID. NO. 066
	1053	CUCCCGU U GUUGGGU	Seq. ID. NO. 067

	1056	CCGUUGU U GGGUAGC	Seq. ID. NO. 068
	1061	GUUGGGU A GCGCUCA	Seq. ID. NO. 069
	1127	GCCACGU C GACUUGC	Seq. ID. NO. 070
	1132	GUCGACU U GCUCGUU	Seq. ID. NO. 071
5	1136	ACUUGCU C GUUGGGG	Seq. ID. NO. 072
	1139	UGCUCGU U GGGGCGG	Seq. ID. NO. 073
	1153	GCCGCUU U CUGUUCC	Seq. ID. NO. 074
	1154	CCGCUUU C UGUUCCG	Seq. ID. NO. 075
	1158	UUUCUGU U CCGCCAU	Seq. ID. NO. 076
10	1159	UUCUGUU C CGCCAUG	Seq. ID. NO. 077
	1168	GCCAUGU A CGUGGGG	Seq. ID. NO. 078
	1189	UGCGGAU C CGUUUUC	Seq. ID. NO. 079
	1193	GAUCCGU U UUCCUCG	Seq. ID. NO. 080
	1194	AUCCGUU U UCCUCGU	Seq. ID. NO. 081
15	1195	UCCGUUU U CCUCGUC	Seq. ID. NO. 082
	1196	CCGUUUU C CUCGUCU	Seq. ID. NO. 083
	1280	GCCAUGU A UCAGGUC	Seq. ID. NO. 084
	1282	CAUGUAU C AGGUCAC	Seq. ID. NO. 085
	1287	AUCAGGU C ACCGCAU	Seq. ID. NO. 086
20	1373	AAGCUGU C GUGGAUA	Seq. ID. NO. 087
	1380	CGUGGAU A UGGUGGC	Seq. ID. NO. 088
	1406	GGGGAGU C CUAGCGG	Seq. ID. NO. 089
	1409	GAGUCCU A GCGGGCC	Seq. ID. NO. 090
	1418	CGGGCCU U GCCUACU	Seq. ID. NO. 091
25	1423	CUUGCCU A CUAUUCC	Seq. ID. NO. 092
	1426	GCCUACU A UUCCAUG	Seq. ID. NO. 093

	1428	CUACUAU U CCAUGGU	Seq. ID. NO. 094
	1429	UACUAUU C CAUGGUG	Seq. ID. NO. 095
	1727	GCUCCAU C GACAAGU	Seq. ID. NO. 096
	1735	GACAAGU U CGCUCAG	Seq. ID. NO. 097
5	1736	ACAAGUU C GCUCAGG	Seq. ID. NO. 098
	1740	GUUCGCU C AGGGAUG	Seq. ID. NO. 099
	1757	GCCCCAU C ACCUAUA	Seq. ID. NO. 100
	1762	AUCACCU A UACCGAG	Seq. ID. NO. 101
	1795	AGGCCUU A CUGCUGG	Seq. ID. NO. 102
10	1806	CUGGCAU U ACGCACC	Seq. ID. NO. 103
	1807	UGGCAUU A CGCACCU	Seq. ID. NO. 104
	1815	CGCACCU C GGCAGUG	Seq. ID. NO. 105
	1827	GUGUGGU A UCGUACC	Seq. ID. NO. 106
	1829	GUGGUAU C GUACCUG	Seq. ID. NO. 107
15	1832	GUAUCGU A CCUGCGU	Seq. ID. NO. 108
	1840	CCUGCGU C GCAGGUG	Seq. ID. NO. 109
	1854	GUGUGGU C CAGUGUA	Seq. ID. NO. 110
	1883	GCCCUGU U GUAGUGG	Seq. ID. NO. 111
	1886	CUGUUGU A GUGGGGA	Seq. ID. NO. 112
20	1902	GACCGAU C GGUCCGG	Seq. ID. NO. 113
	1906	GAUCGGU C CGGUGCC	Seq. ID. NO. 114
	1917	UGCCCCU A CGUAUAA	Seq. ID. NO. 115
	1921	CCUACGU A UAACUGG	Seq. ID. NO. 116
	1923	UACGUAU A ACUGGGG	Seq. ID. NO. 117
25	1990	AACUGGU U UGGCUGU	Seq. ID. NO. 118
	1991	ACUGGUU U GGCUGUA	Seq. ID. NO. 119

	1998	UGGCUGU A CAUGGAU	Seq. ID. NO. 120
	2043	GGGCCCU C CGUGCAA	Seq. ID. NO. 121
	2054	GCAACAU C GGGGGGG	Seq. ID. NO. 122
	2063	GGGGGGU C GGCAACC	Seq. ID. NO. 123
5	2072	GCAACCU C ACCUUGA	Seq. ID. NO. 124
	2077	CUCACCU U GACCUGC	Seq. ID. NO. 125
	2121	GGCCACU U ACACAAA	Seq. ID. NO. 126
	2122	GCCACUU A CACAAAA	Seq. ID. NO. 127
	2137	UGUGGCU C GGGGCCA	Seq. ID. NO. 128
10	2149	CCAUGGU U AACACCU	Seq. ID. NO. 129
	2150	CAUGGUU A ACACCUA	Seq. ID. NO. 130
	2219	UUACCAU C UUUAAGG	Seq. ID. NO. 131
	2221	ACCAUCU U UAAGGUU	Seq. ID. NO. 132
	2261	ACAGGCU U AGUGCUG	Seq. ID. NO. 133
15	2262	CAGGCUU A GUGCUGC	Seq. ID. NO. 134
	2295	AGAGCGU U GCGACCU	Seq. ID. NO. 135
	2320	GACAGAU C GGAGCUC	Seq. ID. NO. 136
	2327	CGGAGCU C AGCCCGC	Seq. ID. NO. 137
	2344	CUGCUGU C CACGACA	Seq. ID. NO. 138
20	2417	UCCACCU C CAUCAGA	Seq. ID. NO. 139
	2421	CCUCCAU C AGAACAU	Seq. ID. NO. 140
	2429	AGAACAU C GUGGACG	Seq. ID. NO. 141
	2534	CGCGCGU C UGUGCCU	Seq. ID. NO. 145
	2585	CCGCCCU A GAGAACC	Seq. ID. NO. 143
25	2600	UGGUGGU C CUCAACG	Seq. ID. NO. 144
	2603	UGGUCCU C AACGCGG	Seq. ID. NO. 145

	2671	GCCUGGU A CAUCAAG	Seq. ID. NO. 146
	2675	GGUACAU C AAGGGCA	Seq. ID. NO. 147
	2690	GGCUGGU C CCUGGGG	Seq. ID. NO. 148
	2704	GCGGCAU A UGCUCUG	Seq. ID. NO. 149
5	2709	AUAUGCU C UGUACGG	Seq. ID. NO. 150
	2713	GCUCUGU A CGGCGUG	Seq. ID. NO. 151
	2738	UCCUGCU C CUGCUGG	Seq. ID. NO. 152
	2763	ACGGGCU U ACGCCAU	Seq. ID. NO. 153
	2764	CGGGCUU A CGCCAUG	Seq. ID. NO. 154
10	2878	UGGUGGU U ACAAUAC	Seq. ID. NO. 155
	2879	GGUGGUU A CAAUACU	Seq. ID. NO. 156
	2884	UUACAAU A CUUUAUC	Seq. ID. NO. 157
	2887	CAAUACU U UAUCACC	Seq. ID. NO. 158
	2888	AAUACUU U AUCACCA	Seq. ID. NO. 159
15	2910	GGCGCAU U UGUGCGU	Seq. ID. NO. 160
	2911	GCGCAUU U GUGCGUG	Seq. ID. NO. 161
	2924	UGUGGGU C CCCCCUC	Seq. ID. NO. 162
	2931	CCCCCCU C UCAAUGU	Seq. ID. NO. 163
	2933	CCCCUCU C AAUGUCC	Seq. ID. NO. 164
20	2939	UCAAUGU C CGGGGGG	Seq. ID. NO. 165
	2958	CGAUGCU A UCAUCCU	Seq. ID. NO. 166
	2960	AUGCUAU C AUCCUCC	Seq. ID. NO. 167
	2963	CUAUCAU C CUCCUCA	Seq. ID. NO. 168
	2966	UCAUCCU C CUCACAU	Seq. ID. NO. 169
25	2969	UCCUCCU C ACAUGUG	Seq. ID. NO. 170
	3059	CUGCCAU A ACUGCGA	Seq. ID. NO. 171

	3138	AGGCCAU U ACGUCCA	Seq. ID. NO. 172
	3139	GGCCAUU A CGUCCAA	Seq. ID. NO. 173
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	3266	CCGU CGU C UUCUCUG	Seq. ID. NO. 182
	3268	GUCGUCU U CUCUGAC	Seq. ID. NO. 183
	3290	CCAAGAU C AUCACCU	Seq. ID. NO. 184
	3293	AGAUCAU C ACCUGGG	Seq. ID. NO. 185
15	3329	GGGACAU C AUCUUGG	Seq. ID. NO. 186
	3332	ACAUCAU C UUGGGAC	Seq. ID. NO. 187
	3334	AUCAUCU U GGGACUG	Seq. ID. NO. 188
	3347	UGCCCGU C UCCGCCC	Seq. ID. NO. 189
	3349	CCCGUCU C CGCCCGA	Seq. ID. NO. 190
20	3371	GGGAGAU A CUUCUGG	Seq. ID. NO. 191
	3416	GGCGACU C CUUGCCC	Seq. ID. NO. 192
	3419	GACUCCU U GCCCCCA	Seq. ID. NO. 193
	3428	CCCCCAU C ACGGCCU	Seq. ID. NO. 194
	3482	CUAGCCU C ACAGGCC	Seq. ID. NO. 195
25	3518	GGGAGGU U CAAGUGG	Seq. ID. NO. 196
	3519	GGAGGUU C AAGUGGU	Seq. ID. NO. 197

	3527	AAGUGGU U UCCACCG	Seq. ID. NO. 198
	3528	AGUGGUU U CCACCGC	Seq. ID. NO. 199
	3529	GUGGUUU C CACCGCA	Seq. ID. NO. 200
	3576	UGUGUGU U GGACCGU	Seq. ID. NO. 201
5	3601	GCCGGCU C AAAGACC	Seq. ID. NO. 202
	3611	AGACCCU A GCCGGCC	Seq. ID. NO. 203
	3684	UGC GCCU C CCGGGGC	Seq. ID. NO. 204
	3696	GGCGCGU U CCCUAC	Seq. ID. NO. 205
	3697	GCGCGUU C CCUACA	Seq. ID. NO. 206
10	3701	GUUCCCU U ACACCAU	Seq. ID. NO. 207
	3702	UUCCCUU A CACCAUG	Seq. ID. NO. 208
	3724	GGUAGCU C GGACCUC	Seq. ID. NO. 209
	3731	CGGACCU C UAUCUGG	Seq. ID. NO. 210
	3733	GACCUCU A UCUGGUC	Seq. ID. NO. 211
15	3735	CCUCUAU C UGGUCAC	Seq. ID. NO. 212
	3740	AUCUGGU C ACGAGAC	Seq. ID. NO. 213
	3761	ACGUCAU U CCGGUGC	Seq. ID. NO. 214
	3762	CGUCAUU C CGGUGCG	Seq. ID. NO. 215
	3786	UGACGGU C GGGGGAG	Seq. ID. NO. 216
20	3797	GGAGCCU A CUGUCCC	Seq. ID. NO. 217
	3802	CUACUGU C CCCCAGA	Seq. ID. NO. 218
	3835	GGCUCUU C GGGUGGC	Seq. ID. NO. 219
	3851	CACUGCU C UGCCC UU	Seq. ID. NO. 220
	3858	CUGCCCU U CGGGGCA	Seq. ID. NO. 221
25	3859	UGCCC UU C GGGGCAC	Seq. ID. NO. 222
	3872	ACGCUGU A GGCAUCU	Seq. ID. NO. 223

	3878	UAGGCAU C UUCCGGG	Seq. ID. NO. 224
	3880	GGCAUCU U CCGGGCU	Seq. ID. NO. 225
	3881	GCAUCUU C CGGGCUG	Seq. ID. NO. 226
	3908	GGGGGGU U GCGAAGG	Seq. ID. NO. 227
5	4056	GAGCACU A AAGUGCC	Seq. ID. NO. 228
	4072	GCUGCGU A CGCAGCC	Seq. ID. NO. 229
	4087	CAAGGGU A CAAGGUA	Seq. ID. NO. 230
	4115	CAUCUGU U GCCGCCA	Seq. ID. NO. 231
	4175	CCAACAU C AGAACUG	Seq. ID. NO. 232
10	4187	CUGGGGU A AGGACCA	Seq. ID. NO. 233
	4228	UCCACCU A UGGUAAG	Seq. ID. NO. 234
	4233	CUAUGGU A AGUUCCU	Seq. ID. NO. 235
	4237	GGUAAGU U CCUUGCC	Seq. ID. NO. 236
	4238	GUAAGUU C CUUGCCG	Seq. ID. NO. 237
15	4241	AGUUCCU U GCCGACG	Seq. ID. NO. 238
	4280	AUAUCAU A AUAUGUG	Seq. ID. NO. 239
	4283	UCAUAAU A UGUGAUG	Seq. ID. NO. 240
	4337	GCACAGU C CUGGACC	Seq. ID. NO. 241
	4370	CGCGGCU C GUCGUGC	Seq. ID. NO. 242
20	4373	GGCUCGU C GUGCUCG	Seq. ID. NO. 243
	4379	UCGUGCU C GCCACCG	Seq. ID. NO. 244
	4425	CCCAAAU A UUGAGGA	Seq. ID. NO. 245
	4444	GCUCUGU C CAACACU	Seq. ID. NO. 246
	4460	GAGAGAU C CCCUUCU	Seq. ID. NO. 243
25	4481	AGGCCAU C CCCUCUG	Seq. ID. NO. 248
	4487	UCCCCCU C GAGGCCA	Seq. ID. NO. 249

	4496	AGGCCAU C AAGGGGG	Seq. ID. NO. 250
	4528	UGCCACU C CAAGAAG	Seq. ID. NO. 251
	4577	UCGGAAU C AAUGCCG	Seq. ID. NO. 252
	4586	AUGCCGU A GCGUAUU	Seq. ID. NO. 253
5	4591	GUAGCGU A UUACCGG	Seq. ID. NO. 254
	4593	AGCGUAU U ACCGGGG	Seq. ID. NO. 255
	4594	GCGUAUU A CCGGGGU	Seq. ID. NO. 256
	4616	UGUCCGU C AUACCGA	Seq. ID. NO. 257
	4619	CCGUCAU A CCGACUA	Seq. ID. NO. 258
10	4626	ACCGACU A GCGGAGA	Seq. ID. NO. 259
	4672	ACGGGCU A CACCGGU	Seq. ID. NO. 260
	4697	CGGUGAU C GACUGCA	Seq. ID. NO. 261
	4789	GCGGUGU C GCGCUCA	Seq. ID. NO. 162
	4795	UCGCGCU C ACAACGG	Seq. ID. NO. 263
15	4920	CUGUGCU U GGUAUGA	Seq. ID. NO. 264
	4924	GCUUGGU A UGAGCUC	Seq. ID. NO. 265
	4931	AUGAGCU C ACGCCCG	Seq. ID. NO. 266
	4947	UGAGACU A CAGUCAG	Seq. ID. NO. 267
	4952	CUACAGU C AGGUUGC	Seq. ID. NO. 268
20	4957	GUCAGGU U GCGGGCU	Seq. ID. NO. 269
	4965	GCGGGCU U ACCUGAA	Seq. ID. NO. 270
	4966	CGGGCUU A CCUGAAU	Seq. ID. NO. 271
	4974	CCUGAAU A CACCAGG	Seq. ID. NO. 272
	4984	CCAGGGU U GCCCCUC	Seq. ID. NO. 273
25	4991	UGCCCGU C UGCCAGG	Seq. ID. NO. 274
	5004	GGACCAU C UGGAGUU	Seq. ID. NO. 275

	5102	ACCUGGU A GCAUACC	Seq. ID. NO. 276
	5107	GUAGCAU A CCAAGCC	Seq. ID. NO. 277
	5133	CAGGGCU C AGGCUC	Seq. ID. NO. 278
	5218	CUGCUGU A UAGGCUA	Seq. ID. NO. 279
5	5220	GCUGUAU A GGCUAGG	Seq. ID. NO. 280
	5306	UGGAGGU C GUCACUA	Seq. ID. NO. 281
	5309	AGGUCGU C ACUAGCA	Seq. ID. NO. 282
	5313	CGUCACU A GCACCUG	Seq. ID. NO. 283
	5330	UGCUGGU A GCGGAG	Seq. ID. NO. 284
10	5339	GCGGAGU C CUUGCAG	Seq. ID. NO. 285
	5342	GAGUCCU U GCAGCUC	Seq. ID. NO. 286
	5359	GCCGCAU A UUGCCUG	Seq. ID. NO. 287
	5361	CGCAUUAU U GCCUGAC	Seq. ID. NO. 288
	5376	AACCGGU A GUGUGGU	Seq. ID. NO. 289
15	5399	GUAGGAU C AUUUUGU	Seq. ID. NO. 290
	5423	CGGCUGU U GUUCCCG	Seq. ID. NO. 291
	5426	CUGUUGU U CCCGACA	Seq. ID. NO. 292
	5427	UGUUGUU C CCGACAG	Seq. ID. NO. 293
	5524	GAGCAGU U CAAGCAG	Seq. ID. NO. 294
20	5525	AGCAGUU C AAGCAGA	Seq. ID. NO. 295
	5583	CGCUGCU C CCGUGGU	Seq. ID. NO. 296
	5596	GUGGAGU C CAGGUGG	Seq. ID. NO. 297
	5612	GGGCCCU U GAGGCCU	Seq. ID. NO. 298
	5620	GAGGCCU U CUGGGCA	Seq. ID. NO. 299
25	5621	AGGCCUU C UGGGCAA	Seq. ID. NO. 300
	5674	GCAGGCU U AUCCACU	Seq. ID. NO. 301

	5675	CAGGCUU A UCCACUC	Seq. ID. NO. 302
	5767	CUCCUGU U CAACAUC	Seq. ID. NO. 303
	5768	UCCUGUU C AACAUUC	Seq. ID. NO. 304
	5801	CUCAACU C GCUCCUC	Seq. ID. NO. 305
5	5805	ACUCGCU C CUCCCAG	Seq. ID. NO. 306
	5821	GCUGCUU C GGCCUUC	Seq. ID. NO. 307
	5827	UCGGCCU U CGUGGGC	Seq. ID. NO. 308
	5828	CGGCCUU C GUGGGCG	Seq. ID. NO. 309
	5843	CCGGCAU U GCCGGUG	Seq. ID. NO. 310
10	5858	CGGCCAU U GGCAGCA	Seq. ID. NO. 311
	5867	GCAGCAU A GGCCUUG	Seq. ID. NO. 312
	5873	UAGGCCU U GGAAGG	Seq. ID. NO. 313
	5905	GCGGGCU A UGGAGCG	Seq. ID. NO. 314
	5930	GUGCACU C GUGGCUU	Seq. ID. NO. 315
15	5937	CGUGGCU U UUAAGGU	Seq. ID. NO. 316
	5938	GUGGCUU U UAAGGUC	Seq. ID. NO. 317
	5939	UGGCUUU U AAGGUCA	Seq. ID. NO. 318
	5940	GGCUUUU A AGGUCAU	Seq. ID. NO. 319
	5945	UUAAGGU C AUGAGCG	Seq. ID. NO. 320
20	5965	GCGCCCU C CGCCGAG	Seq. ID. NO. 321
	5981	ACCUGGU U AACUUGC	Seq. ID. NO. 322
	5982	CCUGGUU A ACUUGC	Seq. ID. NO. 323
	5990	ACUUGC C CCUGCCA	Seq. ID. NO. 324
	6004	AUCCUCU C CCCCGGC	Seq. ID. NO. 325
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	6023	UGGUCGU C GGGGUCG	Seq. ID. NO. 327

	6029	UCGGGGU C GUGUGUG	Seq. ID. NO. 328
	6044	CAGCAAU C CUGCGUC	Seq. ID. NO. 329
	6051	CCUGCGU C GGCACGU	Seq. ID. NO. 340
	6106	AUAGCGU U CGCUUCG	Seq. ID. NO. 341
5	6107	UAGCGUU C GCUUCGC	Seq. ID. NO. 342
	6111	GUUCGCU U CGCGGGG	Seq. ID. NO. 343
	6413	ACGGCAU C AUGCAAA	Seq. ID. NO. 344
	6574	CCGAACU A UUCCAGG	Seq. ID. NO. 345
	6576	GAACUAU U CCAGGGC	Seq. ID. NO. 346
10	6577	AACUAUU C CAGGGCG	Seq. ID. NO. 347
	6637	GGGGACU U CCACUAC	Seq. ID. NO. 348
	6638	GGGACUU C CACUACG	Seq. ID. NO. 349
	6643	UUCCACU A CGUGACG	Seq. ID. NO. 350
	6671	ACAACGU A AAAUGCC	Seq. ID. NO. 351
15	6703	CCCGAAU U CUUCACC	Seq. ID. NO. 352
	6704	CCGAAUU C UUCACCG	Seq. ID. NO. 353
	6706	GAAUUCU U CACCGAA	Seq. ID. NO. 354
	6707	AAUUCUU C ACCGAAU	Seq. ID. NO. 355
	6715	ACCGAAU U GGACGGG	Seq. ID. NO. 356
20	6730	GUGCGGU U GCACAGG	Seq. ID. NO. 357
	6739	CACAGGU A CGCUCCG	Seq. ID. NO. 358
	6744	GUACGCU C CGGCGUG	Seq. ID. NO. 359
	6759	CAGACCU C UCCUACG	Seq. ID. NO. 360
	6761	GACCUCU C CUACGGG	Seq. ID. NO. 361
25	6764	CUCUCCU A CGGGAGG	Seq. ID. NO. 362
	6776	AGGAUGU C ACAUCC	Seq. ID. NO. 363

	6782	UCACAUU C CAGGUUCG	Seq. ID. NO. 364
	6788	UCCAGGU C GGGCUCA	Seq. ID. NO. 365
	6794	UCGGGCU C AACCAAU	Seq. ID. NO. 366
	6802	AACCAAU A CCUGGUU	Seq. ID. NO. 367
5	6809	ACCUGGU U GGGUCAC	Seq. ID. NO. 368
	6814	GUUGGGU C ACAGCUC	Seq. ID. NO. 369
	6821	CACAGCU C CCAUGCG	Seq. ID. NO. 370
	6906	UAAACGU A GGCUGGC	Seq. ID. NO. 371
	6922	AGGGGGU C UCCCCC	Seq. ID. NO. 372
10	6924	GGGGUCU C CCCCCUC	Seq. ID. NO. 373
	6931	CCCCCU C CUUGGCC	Seq. ID. NO. 374
	6934	CCCUCCU U GGCCAGC	Seq. ID. NO. 375
	6943	GCCAGCU C UUCAGCU	Seq. ID. NO. 376
	6958	AGCCAAU U GUCUGCG	Seq. ID. NO. 377
15	6961	CAAUUGU C UGCGCCU	Seq. ID. NO. 378
	7034	CCAACCU C CUGUGGC	Seq. ID. NO. 379
	7118	ACCCGCU U CGAGCGG	Seq. ID. NO. 380
	7119	CCCGCUU C GAGCGGA	Seq. ID. NO. 381
	7145	GGGAAGU A UCCGUUG	Seq. ID. NO. 382
20	7195	CCCGCGU U GCCCAUA	Seq. ID. NO. 383
	7202	UGCCCAU A UGGGCAC	Seq. ID. NO. 384
	7218	CCCGGAU U ACAACCC	Seq. ID. NO. 385
	7219	CCGGAUU A CAACCCU	Seq. ID. NO. 386
	7234	CCACUGU U AGAGUCC	Seq. ID. NO. 387
25	7235	CACUGUU A GAGUCCU	Seq. ID. NO. 388
	7251	GAAAAGU C CGGACUA	Seq. ID. NO. 389

	7258	CCGGACU A CGUCCCU	Seq. ID. NO. 390
	7262	ACUACGU C CCUCCGG	Seq. ID. NO. 391
	7266	CGUCCCU C CGGCGGU	Seq. ID. NO. 392
	7288	UGCCCAU U GCCGCCU	Seq. ID. NO. 393
5	7296	GCCGCCU A CCACGGG	Seq. ID. NO. 394
	7354	ACAGAGU C CACCGUG	Seq. ID. NO. 395
	7386	GCUGGCU A CUAAGAC	Seq. ID. NO. 396
	7389	GGCUACU A AGACUUU	Seq. ID. NO. 397
	7395	UAAGACU U UCGGCAG	Seq. ID. NO. 398
10	7396	AAGACUU U CGGCAGC	Seq. ID. NO. 399
	7397	AGACUUU C GGCAGCU	Seq. ID. NO. 400
	7411	UCCGGAU C GUCGGCC	Seq. ID. NO. 410
	7414	GGAUCGU C GGCCGUU	Seq. ID. NO. 402
	7421	CGGCCGU U GACAGCG	Seq. ID. NO. 403
15	7498	UCGUACU C CUCCAUG	Seq. ID. NO. 404
	7501	UACUCCU C CAUGCCC	Seq. ID. NO. 405
	7514	CCCCCUU U GAGGGGG	Seq. ID. NO. 406
	7539	CCCUGAU C UCAGCGA	Seq. ID. NO. 407
	7541	CUGAUCU C AGCGACG	Seq. ID. NO. 408
20	7552	GACGGGU C UUGGUCU	Seq. ID. NO. 409
	7554	CGGGUCU U GGUCUAC	Seq. ID. NO. 410
	7558	UCUUGGU C UACCGUG	Seq. ID. NO. 411
	7560	UUGGUCU A CCGUGAG	Seq. ID. NO. 412
	7589	ACGACAU C GUCUGCU	Seq. ID. NO. 413
25	7592	ACAUCGU C UGCUGCU	Seq. ID. NO. 414
	7600	UGCUGCU C AAUGUCC	Seq. ID. NO. 415

	7606	UCAAUGU C CUACACA	Seq. ID. NO. 416
	7667	UGCCCAU C AACGCGU	Seq. ID. NO. 417
	7723	ACAACAU C CCGCAGU	Seq. ID. NO. 418
	7775	UGCAAGU C CUGGACG	Seq. ID. NO. 419
5	7789	GACCACU A CCGGGAC	Seq. ID. NO. 420
	7839	UAAGGCU A AACUUCU	Seq. ID. NO. 421
	7847	AACUUCU A UCCGUAG	Seq. ID. NO. 422
	7849	CUUCUAU C CGUAGAA	Seq. ID. NO. 423
	7853	UAUCCGU A GAAGAAG	Seq. ID. NO. 424
10	7894	GCCAAAU C UAAAUUU	Seq. ID. NO. 425
	7896	CAAAUCU A AAUUUGG	Seq. ID. NO. 426
	7900	UCUAAAU U UGGCUAU	Seq. ID. NO. 427
	7901	CUAAAUU U GGCUAUG	Seq. ID. NO. 428
	7906	UUUGGCU A UGGGGCA	Seq. ID. NO. 429
15	7955	ACCACAU C CGCUCCG	Seq. ID. NO. 430
	7960	AUCCGCU C CGUGUGG	Seq. ID. NO. 431
	8075	CUCGCCU U AUCGUAU	Seq. ID. NO. 432
	8076	UCGCCUU A UCGUAUU	Seq. ID. NO. 433
	8078	GCCUUAU C GUAUUCC	Seq. ID. NO. 434
20	8170	UCCUCGU A CGGAUUC	Seq. ID. NO. 435
	8176	UACGGAU U CCAGUAC	Seq. ID. NO. 436
	8182	UUCCAGU A CUCUCCU	Seq. ID. NO. 437
	8187	GUACUCU C CUGGGCA	Seq. ID. NO. 438
	8201	AGCGGGU U GAGUUCC	Seq. ID. NO. 439
25	8206	GUUGAGU U CCUGGUG	Seq. ID. NO. 440
	8207	UUGAGUU C CUGGUGA	Seq. ID. NO. 441

	8227	UGGAAAU C AAAGAAA	Seq. ID. NO. 442
	8357	AGGCCAU A AAGUCGC	Seq. ID. NO. 443
	8362	AUAAAGU C GCUCACG	Seq. ID. NO. 444
	8366	AGUCGCU C ACGGAGC	Seq. ID. NO. 445
5	8378	AGCGGCU C UACAUCG	Seq. ID. NO. 446
	8380	CGGCUCU A CAUCGGG	Seq. ID. NO. 447
	8384	UCUACAU C GGGGGCC	Seq. ID. NO. 448
	8424	CUGCGGU U AUCGCCG	Seq. ID. NO. 449
	8425	UGCGGUU A UCGCCGG	Seq. ID. NO. 450
10	8427	CGGUUAU C GCCGGUG	Seq. ID. NO. 451
	8460	GACGACU A GCUGCGG	Seq. ID. NO. 452
	8508	GGCCUGU C GAGCUGC	Seq. ID. NO. 453
	8522	CAAAGCU C CAGGACU	Seq. ID. NO. 454
	8540	CGAUGCU C GUGAACG	Seq. ID. NO. 454
15	8558	ACGACCU U GUCGUUA	Seq. ID. NO. 455
	8561	ACCUUGU C GUUAUCU	Seq. ID. NO. 456
	8564	UUGUCGU U AUCUGUG	Seq. ID. NO. 457
	8638	AGGUACU C UGCCCCC	Seq. ID. NO. 458
	8671	CCAGAAU A CGACUUG	Seq. ID. NO. 459
20	8698	UCAUGCU C CUCCAAC	Seq. ID. NO. 460
	8701	UGCUCU C CAACGUG	Seq. ID. NO. 461
	8728	GACGCAU C CGGCAAA	Seq. ID. NO. 462
	8774	CCCCCU U GCACGGG	Seq. ID. NO. 463
	8842	AUCAUGU A UGCGCCC	Seq. ID. NO. 464
25	8854	CCCACCU U AUGGGCA	Seq. ID. NO. 465
	8855	CCACCUU A UGGGCAA	Seq. ID. NO. 466

	8871	GAUGAUU U UGAUGAC	Seq. ID. NO. 467
	8880	GAUGACU C ACUUCUU	Seq. ID. NO. 468
	8931	CCUGGAU U GUCAGAU	Seq. ID. NO. 469
	8934	GGAUUGU C AGAUCUA	Seq. ID. NO. 470
5	8939	GUCAGAU C UACGGGG	Seq. ID. NO. 471
	8941	CAGAUUCU A CGGGGCC	Seq. ID. NO. 472
	9065	CAUGCCU C AGGAAAC	Seq. ID. NO. 473
	9074	GGAAACU U GGGGUAC	Seq. ID. NO. 474
	9080	UUGGGGU A CCGCCCU	Seq. ID. NO. 475
10	9088	CCGCCCU U GCGAGUC	Seq. ID. NO. 476
	9095	UGCGAGU C UGGAGAC	Seq. ID. NO. 477
	9119	GAAGUGU C CGCGCUA	Seq. ID. NO. 478
	9126	CCGCGCU A GGCUACU	Seq. ID. NO. 479
	9131	CUAGGCU A CUGUCCC	Seq. ID. NO. 480
15	9136	CUACUGU C CCAAGGG	Seq. ID. NO. 481
	9226	GCCGCGU C CCAGCUG	Seq. ID. NO. 482
	9238	CUGGACU U GUCCAGC	Seq. ID. NO. 483
	9241	GACUUGU C CAGCUGG	Seq. ID. NO. 484
	9250	AGCUGGU U CGUUGCU	Seq. ID. NO. 485
20	9251	GCUGGUU C GUUGCUG	Seq. ID. NO. 486
	9254	GGUUCGU U GCUGGUU	Seq. ID. NO. 487
	9278	GAGACAU A UAUCACA	Seq. ID. NO. 488
	9280	GACAUAU A UCACAGC	Seq. ID. NO. 489
	9282	CAUAUUAU C ACAGCCU	Seq. ID. NO. 490
25	9292	AGCCUGU C UCGUGCC	Seq. ID. NO. 491
	9326	GGUGCCU A CUCCUAC	Seq. ID. NO. 492

	9329	GCCUACU C CUACUUU	Seq. ID. NO. 493
	9332	UACUCCU A CUUUCCG	Seq. ID. NO. 494
	9335	UCCUACU U UCCGUAG	Seq. ID. NO. 495
	9336	CCUACUU U CCGUAGG	Seq. ID. NO. 496
5	9337	CUACUUU C CGUAGGG	Seq. ID. NO. 497
	9341	UUUCCGU A GGGGUAG	Seq. ID. NO. 498
	9347	UAGGGGU A GGCAUCU	Seq. ID. NO. 499
	9353	UAGGCAU C UACCUGC	Seq. ID. NO. 500
	9355	GGCAUCU A CCUGCUC	Seq. ID. NO. 501
10	9362	ACCUGCU C CCCAACC	Seq. ID. NO. 502
	9385	GGGAGCU A AUCACUC	Seq. ID. NO. 503
	9388	AGCUAAU C ACUCCAG	Seq. ID. NO. 504
	9392	AAUCACU C CAGGCCA	Seq. ID. NO. 505
	9402	GGCCAAU A GGCCAUC	Seq. ID. NO. 506

15 ^a Sequences which exhibit perfect homology between the two genomes are highlighted in bold face type.

Methods for preparation of ribozymes useful in this invention are generally described by Draper, entitled, "Method and Reagent For Treatment of Arthritic
20 Conditions", filed November 12, 1993, and assigned U.S. Serial No. 08/152,487, the whole of which is hereby incorporated by reference herein.

In a second related aspect, the invention features a mammalian cell which includes an enzymatic RNA molecule as
25 described above. Preferably, the mammalian cell is a human or other primate cell.

In a third related aspect, the invention features an expression vector which includes nucleic acid encoding the enzymatic RNA molecules described above, located in the
30 vector, e.g., in a manner which allows expression of that enzymatic RNA molecule within a mammalian cell.

In a fourth related aspect, the invention features a method for treatment of an HCV-caused disease by administering to a patient an enzymatic RNA molecule which cleaves HCV RNA in the regions discussed above.

5 The invention provides a class of chemical cleaving agents which exhibit a high degree of specificity for the viral RNA of HCV type infected cells. The ribozyme molecule is preferably targeted to a highly conserved sequence region of HCV such that all types and strains of
10 this virus can be treated with a single ribozyme. Such enzymatic RNA molecules can be delivered exogenously to infected cells. In the preferred hammerhead motif the small size (less than 40 nucleotides, preferably between 32 and 36 nucleotides in length) of the molecule allows
15 the cost of treatment to be reduced compared to other ribozyme motifs.

Synthesis of ribozymes greater than 100 nucleotides in length is very difficult using automated methods, and the therapeutic cost of such molecules is prohibitive.
20 Delivery of ribozymes by expression vectors is primarily feasible using only ex vivo treatments. This limits the utility of this approach. In this invention, small ribozyme motifs (e.g., of the hammerhead structure, shown generally in Fig. 1) are used for exogenous delivery.
25 The simple structure of these molecules also increases the ability of the ribozyme to invade targeted regions of the mRNA structure. Thus, unlike the situation when the hammerhead structure is included within longer transcripts, there are no non-ribozyme flanking sequences
30 to interfere with correct folding of the ribozyme structure or with complementary binding of the ribozyme to the mRNA target region.

The enzymatic RNA molecules of this invention can be used to treat HCV virus infections. Infected animals can
35 be treated at the time of productive infection. This timing of treatment will reduce viral loads in infected cells and disable viral replication in any subsequent

rounds of infection. This is possible because the preferred ribozymes disable those structures required for successful initiation of viral protein synthesis. For treatment of transformed hepatocytes, the methods of this invention allow inhibition of the expression of viral genes thought to cause cell transformation.

The preferred targets of the present invention are advantageous over other targets since they do not act only at the time of viral absorption or genomic replication during infection. In addition, viral particles which are released during a first round of infection in the presence of such ribozymes will still be immunogenic by virtue of having their capsids intact. Thus, one method of this invention allows the creation of defective but immunogenic viral particles, and thus a continued possibility of initiation of an immune response in a treated animal.

In addition, the enzymatic RNA molecules of this invention can be used in vitro in a cell culture infected with HCV viruses to produce viral particles which have intact capsids and defective genomic RNA. These particles can then be used for instigation of immune responses in a prophylactic manner, or as a treatment of infected animals.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing will first briefly be described.

Drawing

Fig. 1 is a diagrammatic representation of a hammerhead motif ribozyme showing stems I, II and III (marked (I), (II) and (III) respectively) interacting with an HCV target region. The 5' and 3' ends of both ribozyme and target are shown. Dashes indicate base-paired nucleotides.

Target Sites

The genome of HCV may be subject to rapid genetic drift by virtue of its RNA content and the nature of errors in genomic replication. Those regions (genes) of the genome which are essential for virus replication, however, are expected to maintain a constant sequence (i.e., are conserved) over extensive periods of time. These regions are preferred target sites in this invention since they are more likely to be conserved between different types or strains of HCV viruses, and thus only one ribozyme is needed to destroy all such viruses. Thus, one ribozyme may be used to target all HCV viruses. We have selected several such regions in the genomes of these viruses, and examined their nucleotide sequences for the presence of regions which may be cleaved by ribozymes targeted to those regions. One region analyzed in detail is the 5' non-translated region; other regions (such as the C or NS1 protein ORFs) can be analyzed in a manner similar to that described below.

Ribozymes targeting selected regions of the HCV genome are preferably chosen to cleave the target RNA in a manner which inhibits translation of the RNA. Genes are selected such that such viral replication is inhibited, e.g., by inhibiting protein synthesis. Selection of effective target sites within these critical regions of viral RNA entails testing the accessibility of the target RNA to hybridization with various oligonucleotide probes. These studies can be performed using RNA probes and assaying accessibility by cleaving the hybrid molecule with RNaseH (see below). Alternatively, such a study can use ribozyme probes designed from secondary structure predictions of the RNAs, and assaying cleavage products by polyacrylamide gel electrophoresis (PAGE), to detect the presence of cleaved and uncleaved molecules.

The following is but one example of a method by which suitable target sites can be identified and is not limiting in this invention. Generally, the method

involves identifying potential cleavage sites for a hammerhead ribozyme, and then testing each of these sites to determine their suitability as targets by ensuring that secondary structure formation is minimal.

5 The mRNA sequences of the virus are folded using RNAfold computer analyses. The regions of the mRNA, in this case the 5' nucleotides of the HCV genome, which are predicted to have weak or non-base paired nucleotides are searched for putative ribozyme recognition motifs. These
10 sites represent the preferred sites for hammerhead or other ribozyme cleavage within these target RNAs.

Short RNA substrates corresponding to each of the gene sites are designed. Each substrate is composed of two to three nucleotides at the 5' and 3' ends that will not base
15 pair with a corresponding ribozyme recognition region. The unpaired regions flank a central region of 12-14 nucleotides to which complementary arms in the ribozyme are designed.

The structure of each substrate sequence is predicted
20 using a PC fold computer program. Sequences which give a positive free energy of binding are accepted. Sequences which give a negative free energy are modified by trimming one or two bases from each of the ends. If the modified sequences are still predicted to have a strong secondary
25 structure, they are rejected.

After substrates are chosen, ribozymes are designed to each of the RNA substrates. Ribozyme folding is also analyzed using PC fold.

Ribozyme molecules are sought which form hammerhead
30 motif stem II (see Fig. 1) regions and contain flanking arms which are devoid of intramolecular base pairing. Often the ribozymes are modified by trimming a base from the ends of the ribozyme, or by introducing additional base pairs in stem II to achieve the desired fold.
35 Ribozymes with incorrect folding are rejected. After substrate/ribozyme pairs are found to contain correct intramolecular structures, the molecules are folded

together to predict intermolecular interactions. A schematic representation of a ribozyme with its coordinate base pairing to its cognate target sequence is shown in Figure 1.

5 Using such analyses, the following predictions of effective target sites in the HCV genomic RNA, based upon computer generated RNA structure analysis, were obtained (see Table 1). The target nucleotide is listed with the genomic nucleotide number (in the HCV genome) given to the
10 left of the sequence. Flanking nucleotides are given for reference.

Those targets thought to be useful as ribozyme targets can be tested to determine accessibility to nucleic acid probes in a ribonuclease H assay (see below). This assay
15 provides a quick test of the use of the target site without requiring synthesis of a ribozyme. It can be used to screen for sites most suited for ribozyme attack.

Synthesis of Ribozymes

Ribozymes useful in this invention can be produced by
20 gene transcription as described by Cech, supra, or by chemical synthesis. Chemical synthesis of RNA is similar to that for DNA synthesis. The additional 2'-OH group in RNA, however, requires a different protecting group strategy to deal with selective 3'-5' internucleotide bond
25 formation, and with RNA susceptibility to degradation in the presence of bases. The recently developed method of RNA synthesis utilizing the t-butyldimethylsilyl group for the protection of the 2' hydroxyl is the most reliable method for synthesis of ribozymes. The method repro-
30 ducibly yields RNA with the correct 3'-5' internucleotide linkages, with average coupling yields in excess of 99%, and requires only a two-step deprotection of the polymer.

A method based upon H-phosphonate chemistry gives a relatively lower coupling efficiency than a method based
35 upon phosphoramidite chemistry. This is a problem for synthesis of DNA as well. A promising approach to scale-

up of automatic oligonucleotide synthesis has been described recently for the H-phosphonates. A combination of a proper coupling time and additional capping of "failure" sequences gave high yields in the synthesis of
5 oligodeoxynucleotides in scales in the range of 14 micromoles with as little as 2 equivalents of a monomer in the coupling step. Another alternative approach is to use soluble polymeric supports (e.g., polyethylene glycols), instead of the conventional solid supports. This method
10 can yield short oligonucleotides in hundred milligram quantities per batch utilizing about 3 equivalents of a monomer in a coupling step.

Various modifications to ribozyme structure can be made to enhance the utility of ribozymes. Such
15 modifications will enhance shelf-life, half-life in vitro, stability, and ease of introduction of such ribozymes to the target site, e.g., to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

20 Exogenous delivery of ribozymes benefits from chemical modification of the backbone, e.g., by the overall negative charge of the ribozyme molecule being reduced to facilitate diffusion across the cell membrane. The present strategies for reducing the oligonucleotide charge
25 include: modification of internucleotide linkages by ethylphosphonates, use of phosphoramidites, linking oligonucleotides to positively charged molecules, and creating complex packages composed of oligonucleotides, lipids and specific receptors or effectors for targeted
30 cells. Examples of such modifications include sulfur-containing ribozymes containing phosphorothioates and phosphorodithioates as internucleotide linkages in RNA. Synthesis of such sulfur-modified ribozymes is achieved by use of the sulfur-transfer reagent, ³H-1,2-benzenedithiol-
35 3-one 1,1-dioxide. Ribozymes may also contain ribose modified ribonucleotides. Pyrimidine analogues are prepared from uridine using a procedure employing

diethylamino sulphur trifluoride (DAST) as a starting reagent. Ribozymes can also be either electrostatically or covalently attached to polymeric cations for the purpose of reducing charge. The polymer can be attached
5 to the ribozyme by simply converting the 3'-end to a ribonucleoside dialdehyde which is obtained by a periodate cleavage of the terminal 2',3'-cis diol system. Depending on the specific requirements for delivery systems, other possible modifications may include different linker arms
10 containing carboxyl, amino or thiol functionalities. Yet further examples include use of methylphosphonates and 2'-o-methylribose and 5' or 3' capping or blocking with m_7GpppG or $m_3^{2,2,7}GpppG$.

For example, a kinased ribozyme is contacted with
15 guanosine triphosphate and guanyltransferase to add a m^3G cap to the ribozyme. After such synthesis, the ribozyme can be gel purified using standard procedure. To ensure that the ribozyme has the desired activity, it may be tested with and without the 5' cap using standard
20 procedures to assay both its enzymatic activity and its stability.

Synthetic ribozymes, including those containing various modifiers, can be purified by high pressure liquid chromatography (HPLC). Other liquid chromatography
25 techniques, employing reverse phase columns and anion exchangers on silica and polymeric supports may also be used.

There follows an example of the synthesis of one ribozyme. A solid phase phosphoramidite chemistry is
30 employed. Monomers used are 2'-tert-butyl-dimethylsilyl cyanoethylphosphoramidities of uridine, N-benzoyl-cytosine, N-phenoxyacetyl adenosine and guanosine (Glen Research, Sterling, VA). Solid phase synthesis is carried out on either an ABI 394 or 380B DNA/RNA synthesizer using
35 the standard protocol provided with each machine. The only exception is that the coupling step is increased from 10 to 12 minutes. The phosphoramidite concentration is

0.1M. Synthesis is done on a 1 μ mole scale using a 1 μ mole RNA reaction column (Glen Research). The average coupling efficiencies are between 97% and 98% for the 394 model, and between 97% and 99% for the 380B model, as determined
5 by a calorimetric measurement of the released trityl cation.

Blocked ribozymes are cleaved from the solid support (e.g., CPG), and the bases and diphosphoester moiety deprotected in a sterile vial by dry ethanolic ammonia (2
10 mL) at 55°C for 16 hours. The reaction mixture is cooled on dry ice. Later, the cold liquid is transferred into a sterile screw cap vial and lyophilized.

To remove the 2'-tert-butyl-dimethylsilyl groups from the ribozyme, the residue is suspended in 1 M tetra-n-butylammonium fluoride in dry THF (TBAF), using a 20 fold
15 excess of the reagent for every silyl group, for 16 hours at ambient temperature (about 15-25°C). The reaction is quenched by adding an equal volume of sterile 1 M triethylamine acetate, pH 6.5. The sample is cooled and
20 concentrated on a SpeedVac to half the initial volume.

The ribozymes are purified in two steps by HPLC on a C4 300 μ 5 mm DeltaPak column in an acetonitrile gradient.

The first step, or "trityl on" step, is a separation of 5'-DMT-protected ribozyme(s) from failure sequences
25 lacking a 5'-DMT group. Solvents used for this step are: A (0.1 M triethylammonium acetate, pH 6.8) and B (acetonitrile). The elution profile is: 20% B for 10 minutes, followed by a linear gradient of 20% B to 50% B over 50 minutes, 50% B for 10 minutes, a linear gradient
30 of 50% B to 100% B over 10 minutes, and a linear gradient of 100% B to 0% B over 10 minutes.

The second step is a purification of a completely deblocked ribozyme by a treatment of 2% trifluoroacetic acid on a C4 300 μ 5 mm DeltaPak column in an acetonitrile
35 gradient. Solvents used for this second step are: A (0.1 M Triethylammonium acetate, pH 6.8) and B (80% acetonitrile, 0.1 M triethylammonium acetate, pH 6.8).

The elution profile is: 5% B for 5 minutes, a linear gradient of 5% B to 15% B over 60 minutes, 15% B for 10 minutes, and a linear gradient of 15% B to 0% B over 10 minutes.

- 5 The fraction containing ribozyme is cooled and lyophilized on a SpeedVac. Solid residue is dissolved in a minimum amount of ethanol and sodium perchlorate in acetone. The ribozyme is collected by centrifugation, washed three times with acetone, and lyophilized.

10 Expression Vector

- While synthetic ribozymes are preferred in this invention, those produced by expression vectors can also be used. In designing a suitable ribozyme expression vector the following factors are important to consider.
- 15 The final ribozyme must be kept as small as possible to minimize unwanted secondary structure within the ribozyme. A promoter (e.g., the human cytomegalovirus immediate early promoter) should be chosen to be a relatively strong promoter, and expressible both in vitro and in vivo. Such
- 20 a promoter should express the ribozyme at a level suitable to effect production of enough ribozyme to destroy a target RNA, but not at too high a level to prevent other cellular activities from occurring (unless cell death itself is desired).

- 25 A hairpin at the 5' end of the ribozyme is useful to protect the ribozyme from 5'-3' exonucleases. A selected hairpin at the 3' end of the ribozyme is useful because it acts as a protection from 3'-5' exonucleases. Such hairpins can be inserted within the vector sequences to
- 30 allow standard ribozymes to be placed in an appropriate orientation and expressed with such sequences attached.

- Poly(A) tails are also useful to protect the 3' end of the ribozyme. These can be provided by either including a poly(A) signal site in the expression vector (to signal
- 35 a cell to add the poly(A) tail in vivo), or by introducing a poly(A) sequence directly into the expression vector.

In the first approach the signal must be located to prevent unwanted secondary structure formation with other parts of the ribozyme. In the second approach, the poly(A) stretch may reduce in size over time when
5 expressed in vivo, and thus the vector may need to be checked over time. Care must be taken in addition of a poly(A) tail which binds poly(A) binding proteins which prevent the ribozyme from acting upon their target sequence.

10 Ribozyme Testing

Once the desired ribozymes are selected, synthesized and purified, they are tested in kinetic and other experiments to determine their utility. An example of such a procedure is provided below.

15 Preparation of Ribozyme

Crude synthetic ribozyme (typically 350 μ g at a time) is purified by separation on a 15% denaturing polyacrylamide gel (0.75 mm thick, 40 cm long) and visualized by UV shadowing. Once excised, gel slices
20 containing full length ribozyme are soaked in 5 ml gel elution buffer (0.5 M NH_4OAc , 1 mM EDTA) overnight with shaking at 4°C. The eluent is desalted over a C-18 matrix (Sep-Pak cartridges, Millipore, Milford, MA) and vacuum dried. The dried RNA is resuspended in 50-100 μ l TE (TRIS
25 10 mM, EDTA 1 mM, pH 7.2). An aliquot of this solution is diluted 100 fold into 1 ml TE, half of which was used to spectrophotometrically quantitate the ribozyme solution. The concentration of this dilute stock is typically 150-800 nM. Purity of the ribozyme is confirmed by the
30 presence of a single band on a denaturing polyacrylamide gel.

A ribozyme may advantageously be synthesized in two or more portions. Each portion of a ribozyme will generally have only limited or no enzymatic activity, and the
35 activity will increase substantially (by at least 5-10

fold) when all portions are ligated (or otherwise juxtaposed) together. A specific example of hammerhead ribozyme synthesis is provided below.

The method involves synthesis of two (or more) shorter
5 "half" ribozymes and ligation of them together using T4
RNA ligase. For example, to make a 34 mer ribozyme, two
17 mers are synthesized, one is phosphorylated, and both
are gel purified. These purified 17 mers are then
annealed to a DNA splint strand complementary to the two
10 17 mers. This DNA splint has a sequence designed to
locate the two 17 mer portions with one end of each
adjacent each other. The juxtaposed RNA molecules are
then treated with T4 RNA ligase in the presence of ATP.
The 34 mer RNA so formed is then HPLC purified.

15 Preparation of Substrates

Approximately 10-30 pmoles of unpurified substrate is
radioactively 5' end-labelled with T4 polynucleotide
kinase using 25 pmoles of [Letter to client re action
proposed γ -³²P] ATP. The entire labelling mix is separated
20 on a 20% denaturing polyacrylamide gel and visualized by
autoradiography. The full length band is excised and
soaked overnight at 4°C in 100 μ l of TE (10 mM Tris-HCl pH
7.6, 0.1 mM EDTA).

Kinetics Reactions

25 For reactions using short substrates (between 8 and 16
bases) a substrate solution is made 1X in assay buffer (75
mM Tris-HCl, pH 7.6; 0.1 mM EDTA, 10 mM MgCl₂) such that
the concentration of substrate is less than 1nM. A
ribozyme solution (typically 20 nM) is made 1X in assay
30 buffer and four dilutions are made using 1X assay buffer.
Fifteen μ l of each ribozyme dilution (i.e., 20, 16, 12, 8
and 4 nM) is placed in a separate tube. These tubes and
the substrate tube are pre-incubated at 37°C for at least
five minutes.

The reaction is started by mixing 15 μ l of substrate into each ribozyme tube by rapid pipetting (note that final ribozyme concentrations are 10, 8, 6, 4, 2 nM). 5 μ l aliquots are removed at 15 or 30 second intervals and quenched with 5 μ l stop solution (95% formamide, 20 mM EDTA xylene cyanol, and bromphenol blue dyes). Following the final ribozyme time point, an aliquot of the remaining substrate is removed as a zero ribozyme control.

The samples are separated on either 15% or 20% polyacrylamide gels. Each gel is visualized and quantitated with an Ambis beta scanner (Ambis Systems, San Diego, CA).

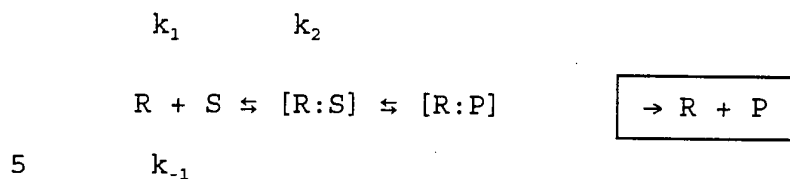
For the most active ribozymes, kinetic analyses are performed in substrate excess to determine K_m and K_{cat} values.

For kinetic reactions with long RNA substrates (greater than 15 bases in length) the substrates are prepared by transcription using T7 RNA polymerase and defined templates containing a T7 promoter, and DNA encoding appropriate nucleotides of the viral RNA. The substrate solution is made 1X in assay buffer (75 mM Tris-HCl, pH 7.6; 0.1 mM EDTA; 10 mM $MgCl_2$) and contains 58 nanomolar concentration of the long RNA molecules. The reaction is started by addition of gel purified ribozymes to 1 μ M concentration. Aliquots are removed at 20, 40, 60, 80 and 100 minutes, then quenched by the addition of 5 μ l stop solution. Cleavage products are separated using denaturing PAGE. The bands are visualized and quantitated with an Ambis beta scanner.

30 Kinetic Analysis

A simple reaction mechanism for ribozyme-mediated cleavage is:

40



where R = ribozyme, S = substrate, and P = products. The boxed step is important only in substrate excess. Because ribozyme concentration is in excess over substrate concentration, the concentration of the ribozyme-substrate complex ([R:S]) is constant over time except during the very brief time when the complex is being initially formed, i.e.,:

$$\frac{d[R:S]}{dt} = 0$$

where t = time, and thus:

$$(R)(S)k_1 = (RS)(k_2 + k_{-1}).$$

The rate of the reaction is the rate of disappearance of substrate with time:

$$\text{Rate} = \frac{-d(S)}{dt} = k_2(RS)$$

Substituting these expressions:

$$(R)(S)k_1 = 1/k_2 \frac{-d(S)}{dt} (k_2 + k_{-1})$$

or:

$$\frac{-d(S)}{S} = \frac{k_1 k_2}{(k_2 + k_{-1})} (R) dt$$

Integrating this expression with respect to time yields:

$$-\ln \frac{S}{S_0} = \frac{k_1 k_2}{(k_2 + k_{-1})} (R) t$$

where S_0 = initial substrate. Therefore, a plot of the negative log of fraction substrate uncut versus time (in minutes) yields a straight line with slope:

41

$$\text{slope} = \frac{k_1 k_2}{(k_2 + k_1)} (R) = k_{\text{obs}}$$

where k_{obs} = observed rate constant. A plot of slope (k_{obs}) versus ribozyme concentration yields a straight line
5 with a slope which is:

$$\text{slope} = \frac{k_1 k_2}{(k_2 + k_1)} \text{ which is } \frac{k_{\text{cat}}}{K_m}$$

Using these equations the data obtained from the kinetic experiments provides the necessary information to
10 determine which ribozyme tested is most useful, or active. Such ribozymes can be selected and tested in in vivo or ex vivo systems.

Liposome Preparation

Lipid molecules are dissolved in a volatile organic
15 solvent (CHCl_3 , methanol, diethylether, ethanol, etc.). The organic solvent is removed by evaporation. The lipid is hydrated into suspension with 0.1x phosphate buffered saline (PBS), then freeze-thawed 3x using liquid nitrogen and incubation at room temperature. The suspension is
20 extruded sequentially through a 0.4 μm , 0.2 μm and 0.1 μm polycarbonate filters at maximum pressure of 800 psi. The ribozyme is mixed with the extruded liposome suspension and lyophilized to dryness. The lipid/ribozyme powder is rehydrated with water to one-tenth the original volume.
25 The suspension is diluted to the minimum volume required for extrusion (0.4ml for 1.5ml barrel and 1.5ml for 10ml barrel) with 1xPBS and re-extruded through 0.4 μm , 0.2 μm , 0.1 μm polycarbonate filters. The liposome entrapped ribozyme is separated from untrapped ribozyme by gel
30 filtration chromatography (SEPHAROSE CL-4B, BIOGEL A5M). The liposome extractions are pooled and sterilized by filtration through a 0.2 μm filter. The free ribozyme is pooled and recovered by ethanol precipitation. The liposome concentration is determined by incorporation of
35 a radioactive lipid. The ribozyme concentration is determined by labeling with ^{32}P . Rossi et al., 1992 supra

(and references cited therein) describe other methods suitable for preparation of liposomes.

In Vivo Assay

The efficacy of action of a chosen ribozyme may be tested in vivo by use of cell cultures sensitive to HCV using standard procedures. For example, monolayer cultures of HCV-sensitive HepG2 cells are grown in 6 or 96 well tissue culture plates. Prior to transduction with HCV expression plasmids, cultures are treated for three to 24 hours with ribozyme-containing liposomes or cationic lipid/ribozyme complexes. Cells are then rinsed with phosphate buffered saline (PBS) and transduced or transfected with HCV expression plasmids. The cells are treated for three to five days with appropriate ribozyme preparations in fresh changes of medium. Total cellular RNA is harvested by the guanidine isothiocyanate technique and the amount of HCV mRNA is quantified and assessed for ribozyme-mediated cleavage using the ribonuclease protection assay. Alternatively, cellular lysates can be prepared and the core particles of HCV could be immunoprecipitated using polyclonal anti-core antiserum adsorbed to protein A-Sepharose according to manufacturers directions. The precipitated cores are treated with ribonuclease to digest any non-encapsidated RNAs and the core protein is digested with proteinase K/phenol extraction. Usually one-half of the total RNA and one-half of the extracted RNA is analyzed using the ribonuclease protection assay.

Ribonuclease Protection Assay

The accumulation of target mRNA in cells or the cleavage of the RNA by ribozymes or RNaseH (in vitro or in vivo) can be quantified using an RNase protection assay.

In this method, antisense riboprobes are transcribed from template DNA using T7 RNA polymerase (U.S. Biochemicals) in 20 μ l reactions containing 1X

transcription buffer (supplied by the manufacturer), 0.2mM ATP, GTP and UTP, 1 U/ μ l pancreatic RNase inhibitor (Boehringer Mannheim Biochemicals) and 200 μ Ci ^{32} P-labeled CTP (800 Ci/mmol, New England Nuclear) for 1 h at 37°C.

- 5 Template DNA is digested with 1 U RNase-free DNase I (U.S. Biochemicals, Cleveland, OH) at 37°C for 15 minutes and unincorporated nucleotides removed by G-50 SEPHADEX spin chromatography.

- 10 In a manner similar to the transcription of antisense probe, the target RNA can be transcribed in vitro using a suitable DNA template. The transcript is purified by standard methods and digested with ribozyme at 37°C according to methods described later.

- Alternatively, virus-infected cells are harvested into
15 1 ml of PBS, transferred to a 1.5 ml EPPENDORF tube, pelleted for 30 seconds at low speed in a microcentrifuge, and lysed in 70 μ l of hybridization buffer (4 M guanidine isothiocyanate, 0.1% sarcosyl, 25 mM sodium citrate, pH 7.5). Cell lysate (45 μ l) or defined amounts of in vitro
20 transcript (also in hybridization buffer) is then combined with 5 μ l of hybridization buffer containing 5×10^5 cpm of each antisense riboprobe in 0.5 ml EPPENDORF tubes, overlaid with 25 μ l mineral oil, and hybridization accomplished by heating overnight at 55°C. The
25 hybridization reactions are diluted into 0.5 ml RNase solution (20 U/ml RNase A, 2 U/ml RNase T1, 10 U/ml RNase-free DNase I in 0.4 M NaCl), heated for 30 minutes at 37°C, and 10 μ l of 20% SDS and 10 μ l of Proteinase K (10 mg/ml) added, followed by an additional 30 minutes
30 incubation at 37°C. Hybrids are partially purified by extraction with 0.5 ml of a 1:1 mixture of phenol/chloroform; aqueous phases are combined with 0.5 ml isopropanol, and RNase-resistant hybrids pelleted for 10 minutes at room temperature (about 20°C) in a
35 microcentrifuge. Pellets are dissolved in 10 μ l loading buffer (95% formamide, 1X TBE, 0.1% bromophenol blue, 0.1% xylene cyanol), heated to 95°C for five minutes, cooled

on ice, and analyzed on 4% polyacrylamide/7 M urea gels under denaturing conditions.

Ribozyme Stability

The chosen ribozyme can be tested to determine its stability, and thus its potential utility. Such a test can also be used to determine the effect of various chemical modifications (e.g., addition of a poly(A) tail) on the ribozyme stability and thus aid selection of a more stable ribozyme. For example, a reaction mixture contains 1 to 5 pmoles of 5' (kinased) and/or 3' labeled ribozyme, 15 μ g of cytosolic extract and 2.5 mM $MgCl_2$ in a total volume of 100 μ l. The reaction is incubated at 37°C. Eight μ l aliquots are taken at timed intervals and mixed with 8 μ l of a stop mix (20 mM EDTA, 95% formamide). Samples are separated on a 15% acrylamide sequencing gel, exposed to film, and scanned with an Ambis.

A 3'-labelled ribozyme can be formed by incorporation of the ^{32}P -labeled cordycepin at the 3' OH using poly(A) polymerase. For example, the poly(A) polymerase reaction contains 40 mM Tris, pH 8, 10 mM $MgCl_2$, 250 mM NaCl, 2.5 mM $MnCl_2$,; 3 μ l P^{32} cordycepin, 500 Ci/mM; and 6 units poly(A) polymerase in a total volume of 50 μ l. The reaction mixture was incubated for 30 minutes at 37°C.

Effect of base substitution upon Ribozyme Activity

To determine which primary structural characteristics could change ribozyme cleavage of substrate, minor base changes can be made in the substrate cleavage region recognized by a specific ribozyme. For example, the substrate sequences can be changed at the central "C" nucleotide, changing the cleavage site from a GUC to a GUA motif. The K_{cat}/K_m values for cleavage using each substrate are then analyzed to determine if such a change increases ribozyme cleavage rates. Similar experiments can be performed to address the effects of changing bases complementary to the ribozyme binding arms. Changes

predicted to maintain strong binding to the complementary substrate are preferred. Minor changes in nucleotide content can alter ribozyme/substrate interactions in ways which are unpredictable based upon binding strength alone.

- 5 Structures in the catalytic core region of the ribozyme recognize trivial changes in either substrate structure or the three dimensional structure of the ribozyme/substrate complex.

To begin optimizing ribozyme design, the cleavage
10 rates of ribozymes containing varied arm lengths, but targeted to the same length of short RNA substrate can be tested. Minimal arm lengths are required and effective cleavage varies with ribozyme/substrate combinations.

The cleavage activity of selected ribozymes can be
15 assessed using HCV-homologous substrates or HCV genomic RNA. The assays are performed in ribozyme excess and approximate K_{cat}/K_{min} values obtained. Comparison of values obtained with short and long substrates indicates utility in vivo of a ribozyme.

20 Intracellular Stability of liposome-delivered ribozymes

To test the stability of a chosen ribozyme in vivo the following test is useful. Ribozymes are ^{32}P -end labeled, entrapped in liposomes and delivered to HCV sensitive cells for three hours. The cells are fractionated and
25 purified by phenol/chloroform extraction. Cells (1×10^7 , T-175 flask) are scraped from the surface of the flask and washed twice with cold PBS. The cells are homogenized by douncing 35 times in 4 ml of TSE (10 mM Tris, pH 7.4, 0.25 M Sucrose, mM EDTA). Nuclei are pelleted at 100xg for 10
30 minutes. Subcellular organelles (the membrane fraction) are pelleted at 200,000xg for two hours using an SW60 rotor. The pellet is resuspended in 1 ml of H buffer (0.25 M Sucrose, 50 mM HEPES, pH 7.4). The supernatant contains the cytoplasmic fraction (in approximately 3.7
35 ml). The nuclear pellet is resuspended in 1 ml of 65% sucrose in TM (50 mM Tris, pH 7.4., 2.5 mM $MgCl_2$) and banded

on a sucrose step gradient (1 ml nuclei in 65% sucrose TM, 1 ml 60% sucrose TM, 1 ml 55% sucrose TM, 50% sucrose TM, 300 ul 25% sucrose TM) for one hour at 37,000xg with an SW60 rotor. The nuclear band is harvested and diluted to 10% sucrose with TM buffer. Nuclei are pelleted at 37,000xg using an SW60 rotor for 15 minutes and the pellet resuspended in 1 ml of TM buffer. Aliquots are size fractionated on denaturing polyacrylamide gels and the intracellular localization determined. By comparison to the migration rate of newly synthesized ribozyme, the various fraction containing intact ribozyme can be determined.

To investigate modifications which would lengthen the half-life of ribozyme molecules intracellularly, the cells may be fractionated as above and the purity of each fraction assessed by assaying enzyme activity known to exist in that fraction.

The various cell fractions are frozen at -70°C and used to determine relative nuclease resistances of modified ribozyme molecules. Ribozyme molecules may be synthesized with 5 phosphorothioate (ps), or 2'-O-methyl (2'-OMe) modifications at each end of the molecule. These molecules and a phosphodiester version of the ribozyme are end-labeled with ³²P and ATP using T4 polynucleotide kinase. Equal concentrations are added to the cell cytoplasmic extracts and aliquots of each taken at 10 minute intervals. The samples are size fractionated by denaturing PAGE and relative rates of nuclease resistance analyzed by scanning the gel with an Ambis scanner. The results show whether the ribozymes are digested by the cytoplasmic extract, and which versions are relatively more nuclease resistant. Modified ribozymes generally maintain 80-90% of the catalytic activity of the native ribozyme when short RNA substrates are employed.

Unlabeled, 5' end-labeled or 3' end-labeled ribozymes can be used in the assays. These experiments can also be

performed with human cell extracts to verify the observations.

Administration of Ribozyme

Selected ribozymes can be administered prophylactically, or to virus infected patients, e.g., by exogenous delivery of the ribozyme to an infected tissue by means of an appropriate delivery vehicle, e.g., a liposome, a controlled release vehicle, by use of iontophoresis, electroporation or ion paired molecules, or covalently attached adducts, and other pharmacologically approved methods of delivery. Routes of administration include intramuscular, aerosol, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal. Expression vectors for immunization with ribozymes and/or delivery of ribozymes are also suitable. See Draper, U.S. Serial No. 08/152,487, filed November 12, 1993, the whole of which including drawings is hereby incorporated herein by reference. See, also, Sullivan, U.S. Serial No. 08/155,474, the whole of which including drawings is hereby incorporated herein by reference.

The specific delivery route of any selected ribozyme will depend on the use of the ribozyme. Generally, a specific delivery program for each ribozyme will focus on naked ribozyme uptake with regard to intracellular localization, followed by demonstration of efficacy. Alternatively, delivery to these same cells in an organ or tissue of an animal can be pursued. Uptake studies will include uptake assays to evaluate cellular ribozyme uptake, regardless of the delivery vehicle or strategy. Such assays will also determine the intracellular localization of the ribozyme following uptake, ultimately establishing the requirements for maintenance of steady-state concentrations within the cellular compartment containing the target sequence (nucleus and/or cytoplasm). Efficacy and cytotoxicity can then be tested. Toxicity

will not only include cell viability but also cell function.

Some methods of delivery that may be used include:

- a. encapsulation in liposomes,
- 5 b. transduction by retroviral vectors,
- c. conjugation with cholesterol,
- d. localization to nuclear compartment
utilizing antigen binding site found on most snRNAs,
- e. neutralization of charge of ribozyme by
10 using nucleotide derivatives, and
- f. use of blood stem cells to distribute
ribozymes throughout the body.

At least three types of delivery strategies are useful in the present invention, including: ribozyme
15 modifications, particle carrier drug delivery vehicles, and viral expression vectors. Unmodified ribozymes and antisense oligonucleotides, like most small molecules, are taken up by cells, albeit slowly. To enhance cellular uptake, the ribozyme may be modified essentially at
20 random, in ways which reduces its charge but maintains specific functional groups. This results in a molecule which is able to diffuse across the cell membrane, thus removing the permeability barrier.

Modification of ribozymes to reduce charge is just one
25 approach to enhance the cellular uptake of these larger molecules. The random approach, however, is not advisable since ribozymes are structurally and functionally more complex than small drug molecules. The structural requirements necessary to maintain ribozyme catalytic
30 activity are well understood by those in the art. These requirements are taken into consideration when designing modifications to enhance cellular delivery. The modifications are also designed to reduce susceptibility to nuclease degradation. Both of these characteristics
35 should greatly improve the efficacy of the ribozyme. Cellular uptake can be increased by several orders of

magnitude without having to alter the phosphodiester linkages necessary for ribozyme cleavage activity.

Chemical modifications of the phosphate backbone will reduce the negative charge allowing free diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology. The similarities in chemical composition between DNA and RNA make this a feasible approach. In the body, maintenance of an external concentration will be necessary to drive the diffusion of the modified ribozyme into the cells of the tissue. Administration routes which allow the diseased tissue to be exposed to a transient high concentration of the drug, which is slowly dissipated by systemic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the ribozyme can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made to accumulate at the site of infection, can protect the ribozyme from degradative processes.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

From this category of delivery systems, liposomes are preferred. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity.

Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in
5 size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver RNA to cells and that the RNA remains biologically active.

For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, has been shown to
10 deliver intact mRNA molecules to cells yielding production of the corresponding protein.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be
15 readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

20 Other controlled release drug delivery systems, such as nonoparticles and hydrogels may be potential delivery vehicles for a ribozyme. These carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals, and consequently, can be adapted for
25 ribozyme delivery.

Topical administration of ribozymes is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the ribozyme to the
30 disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the ribozyme to diffuse into the infected cells. Chemical
35 modification of the ribozyme to neutralize negative charge may be all that is required for penetration. However, in the event that charge neutralization is insufficient, the

modified ribozyme can be co-formulated with permeability enhancers, such as Azone or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified ribozyme and permeability enhancer transfer from the liposome into the infected cell, or the liposome phospholipids can participate directly with the modified ribozyme and permeability enhancer in facilitating cellular delivery. In some cases, both the ribozyme and permeability enhancer can be formulated into a suppository formulation for slow release.

Ribozymes may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. Each of these administration routes expose the ribozyme to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the ribozyme at the lymph node. The ribozyme can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified ribozyme to the cell.

Intraperitoneal administration also leads to entry into the circulation, with once again, the molecular weight or size controlling the rate of entry.

Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The rest is left to circulate in the blood stream for up to 24 hours.

The chosen method of delivery should result in cytoplasmic accumulation and molecules should have some nuclease-resistance for optimal dosing. Nuclear delivery may be used but is less preferable. Most preferred
5 delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, microinjection or electroporation (for ex vivo treatments) and other pharmaceutically applicable vehicles. The dosage will depend upon the disease indication and the route of
10 administration but should be between 100-200 mg/kg of body weight/day. The duration of treatment will extend through the course of the disease symptoms, usually at least 14-16 days and possibly continuously. Multiple daily doses are anticipated for topical applications, ocular applications
15 and vaginal applications. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

Establishment of therapeutic levels of ribozyme within the cell is dependent upon the rate of uptake and
20 degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the ribozyme. Thus, chemically modified ribozymes, e.g., with modification of the phosphate backbone, or capping of the 5' and 3' ends of the ribozyme with nucleotide analogs may
25 require different dosaging. Descriptions of useful systems are provided in the art cited above, all of which is hereby incorporated by reference herein.

The claimed ribozymes are also useful as diagnostic tools to specifically or non-specifically detect the
30 presence of a target RNA in a sample. That is, the target RNA, if present in the sample, will be specifically cleaved by the ribozyme, and thus can be readily and specifically detected as smaller RNA species. The presence of such smaller RNA species is indicative of the
35 presence of the target RNA in the sample.

Other embodiments are within the following claims.

Claims

1. An enzymatic RNA molecule which is active to specifically cleave RNA of HCV.
2. The enzymatic RNA molecule of claim 1 which
5 cleaves RNA of HCV in the 5' non-translated region, RNA encoding the core (c) region or the proteases NS3 or NS2/NS3.
3. The enzymatic RNA molecule of claim 1 wherein said RNA molecule is in a hammerhead motif.
- 10 4. The enzymatic RNA molecule of claim 1 wherein said RNA molecule is in a hairpin, hepatitis Delta virus, group 1 intron, or RNaseP RNA motif.
5. The enzymatic RNA molecule of claim 1 which cleaves the sequence shown as any of SEQ. ID. NOS. 1-8.
- 15 6. A mammalian cell including an enzymatic RNA molecule of any of claims 1-5.
7. The cell of claim 6, wherein said cell is a human cell.
8. An expression vector including nucleic acid
20 encoding the enzymatic RNA molecule of any of claims 1-5, in a manner which allow expression of that enzymatic RNA molecule within a mammalian cell.
9. A method for treatment of a disease caused by an HCV or expression of an HCV gene or portion of an HCV gene
25 by administering to a patient an enzymatic RNA molecule of any of claims 1-5.
10. The method of claim 9 wherein said patient is a human, or non-human primate.

11. A method for providing defective viral particles comprising the step of contacting a cell infected with an HCV with an enzymatic RNA molecule active to cleave a gene required for viral replication.

5 12. The method of claim 11 wherein said molecule is active to cleave a nucleic acid required for viral protein synthesis.

13. The method of claim 11 wherein said nucleic acid is in the 5' non-translated region of HCV.

10 14. Defective viral particles produced by contacting a cell infected with an HCV with an enzymatic RNA molecule active to cleave a gene required for viral replication.

15 15. A method for inducing an immune response or for inducing production of anti-HCV immunoglobulin in a human, comprising the step of administering a defective viral particle produced by contacting a cell infected with an HCV with an enzymatic RNA molecule active to cleave a gene required for viral replication.

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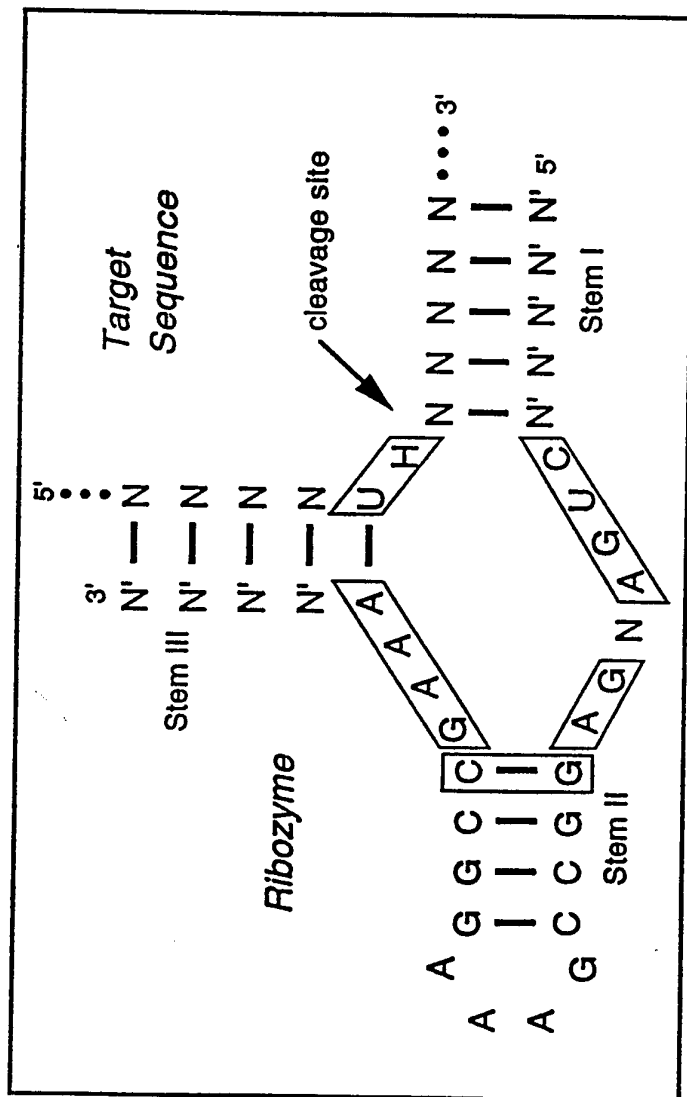


FIG. 1.