TITLE

CONSERVED HBV AND HCV SEQUENCES USEFUL FOR GENE SILENCING

FIELD OF THE INVENTION

This invention relates to methods and compositions utilizing conserved genetic sequences of known hepatitis B viral (HBV) strains and known hepatitis C viral (HCV) strains to modulate the expression of HBV and/or HCV in mammalian cells, via double-stranded RNA-mediated gene silencing, including post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS).

BACKGROUND OF THE INVENTION

Human hepatitis C (HCV) is a major public health problem with an estimated 200 million persons worldwide infected. The number of new infections per year in the United States is estimated to be about 25,000 in 2001. This number has declined from an estimated 240,000 new cases per year in the 1980's due to blood donor screening. Nevertheless, an estimated 3.9 million (1.8%) Americans have been infected with HCV, of whom 2.7 million are chronically infected. Hepatitis C shows significant genetic variation in worldwide populations, evidence of its frequent rates of mutation and rapid evolution. There are six basic genotypes of HCV, with 15 recorded subtypes, which vary in prevalence across different regions of the world. Each of these major genotypes may differ significantly in their biological effects - in terms of replication, mutation rates, type and severity of liver damage, and detection and treatment options - however, these differences are not yet clearly understood.

There is currently no vaccine against HCV and available drug therapy, including ribavirin and interferon, is only partially effective. It is estimated that 75-85% of infected persons will develop a chronic infection, with 70% of chronically infected persons expected to develop chronic liver disease including hepatocellular carcinoma. Chronic HCV related liver disease is a leading indication for liver transplant.

Although a human hepatitis B vaccine has been available since 1982, it is estimated that 350 million persons worldwide are chronically infected with HBV. While the number of new infections per year in the United States has declined from an average of 260,000 in the 1980s to about 78,000 in 2001, there are an estimated 1.25 million hepatitis B carriers, defined as persons positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Such carriers of HBV are at increased risk for developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma. Although most carriers do not develop hepatic complications from chronic hepatitis B, 15% to 40% will develop serious sequelae during their lifetime, and death from chronic liver disease occurs in 15-25% of chronically infected persons.

There is a need for improved therapeutic agents effective in patients suffering from HBV and/or HCV infection, especially chronic infection, which together are estimated to account for 75% of all cases of liver disease around the world. There is also an extreme need for prophylactic methods and agents effective against HCV.

Nucleic acids (e.g., DNA, RNA, hybrid, heteroduplex, and modified nucleic acids) have come to be recognized as extremely valuable agents with significant and varied biological activities, including their use as therapeutic moieties in the prevention and/or treatment of disease states in man and animals. For example, oligonucleotides acting through antisense mechanisms are designed to hybridize to target mRNAs, thereby modulating the activity of the mRNA. Another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of triplex or triple strand formation, in which a single-stranded oligomer (e.g., DNA or

RNA) is designed to bind to a double-stranded DNA target to produce a desired result, e.g., inhibition of transcription from the DNA target. Yet another approach to the utilization of nucleic acids as therapeutics is designed to take advantage of ribozymes, in which a structured RNA or a modified oligomer is designed to bind to an RNA or a double-stranded DNA target to produce a desired result, e.g., targeted cleavage of RNA or the DNA target and thus inhibiting its expression. Nucleic acids may also be used as immunizing agents, e.g., by introducing DNA molecules into the tissues or cells of an organism that express proteins capable of eliciting an immune response. Nucleic acids may also be engineered to encode an RNA with antisense, ribozyme, or triplex activities, or to produce RNA that is translated to produce protein(s) that have biological function.

More recently, the phenomenon of RNAi or double-stranded RNA (dsRNA)-mediated gene silencing has been recognized, whereby dsRNA complementary to a region of a target gene in a cell or organism inhibits expression of the target gene (see, e.g., WO 99/32619, published 1 July 1999, Fire et al.; and U.S. 6,506,559: "Genetic Inhibition by Double-Stranded RNA;" WO 00/63364: "Methods and Compositions for Inhibiting the Function of Polynucleotide Sequences," Pachuk and Satishchandran; and U.S.S.N. 60/419,532, filed October 18, 2002). dsRNA-mediated gene silencing, utilizing compositions providing an at least partially double-stranded dsRNA, is expected to provide extremely valuable therapeutic and/or prophylactic agents against viral infection, including HBV and/or HCV, including in the extremely difficult problem of chronic HBV and/or HCV infection.

SUMMARY OF THE INVENTION

Applicants' invention provides a method for inhibiting expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide

sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. In a preferred embodiment of the method, effector sequences from more than one SEQ ID sequence may be administered to the same cell, and/or more than one effector sequence from within the same SEQ ID sequence may be administered to the same cell.

Applicants' method further provides a method for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising administering to said cell a double-stranded RNA effector molecule comprising comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. In a preferred embodiment of this aspect of the method, effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecule from within the same SEQ ID NO may be administered to the same cell.

Applicants' method further provides A method for inhibiting expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell, comprising administering to said cell a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. In preferred embodiments of this aspect of the invention, effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10

may be administered to the same cell; and/or effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 may be administered to the same cell; and/or more than one effector molecules from within the same SEQ ID NO may be administered to the same cell.

Applicants' invention further provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO is present in the composition.

Applicants' invention further provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector molecule from within the same SEQ ID NO may be present in the composition.

Applicants' invention further provides a composition for inhibiting the expression of both a polynucleotide sequence of hepatitis B virus and a polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID

NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T. Preferred embodiments of the composition include wherein effector molecules from more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition; and/or wherein effector molecules from both SEQ ID NO:11 and SEQ ID NO:12 are present in the composition; and/or wherein more than one effector sequence from within the same SEQ ID NO may be present in the composition.

In particularly preferred embodiments of the above methods and compositions of the invention, the polynucleotide sequence is RNA, and the mammalian cell is a human cell.

Further provided are compositions for inhibiting the expression of a polynucleotide sequence of hepatitis B virus and/or a polynucleotide sequence of hepatitis C virus in mammalian cells, wherein said compositions comprise an at least 19 contiguous nucleotide sequence selected from within SEQ ID NO:1 through SEQ ID NO:12, the complement sequences of SEQ ID NO:1 through SEQ ID NO:12, and mixtures of these sequences. In this embodiment of the invention, the "an at least 19 contiguous nucleotide sequence" is preferably DNA, and the mammalian cell is preferably human. Also provided are expression constructs comprising any of the aforementioned compositions and a mammalian cell comprising said expression constructs.

Another aspect of the invention provides for a polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:26. Another aspect of the invention provides for a polynucleotide

sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

Another aspect of the invention provides a composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 through SEQ ID NO:10 represent conserved regions of the hepatitis B genome.

SEQ ID NO:11 and SEQ ID NO:12 represent conserved regions of the hepatitis C genome.

SEQ ID NO:13 represents the nucleotide sequence of human U6 promoter.

SEQ ID NO:14 and SEQ ID NO:15 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:5.

SEQ ID NO:16 and SEQ ID NO:17 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:4.

SEQ ID NO:18 represents eiRNA that has an HBV sequence mapping within SEQ ID NO:10.

SEQ ID NO:19 through SEQ ID NO:22 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:3.

SEQ ID NO:23 and SEQ ID NO:24 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:2.

SEQ ID NO:25 and SEQ ID NO:26 represent eiRNAs that have HBV sequences mapping within SEQ ID NO:1.

SEQ ID NO:27 represents the "X" region of the HCV 3'UTR.

SEQ ID NO:28 through SEQ ID NO:36 represent siRNAs mapping to the the HCV 3'UTR.

SEQ ID NO:37 through SEQ ID NO:44 represent siRNAs mapping to the "X" region of the HCV 3'UTR.

SEQ ID NO:45 represents an siRNA mapping to the HCV core.

SEQ ID NO:46 represents an siRNA mapping to lamin.

SEQ ID NO:47 represents the T7 RNA polymerase gene.

SEQ ID NO:48 represents a T7-based eiRNA vector encoding a hairpin RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a vector illustrating placement of the T7 RNA polymerase promoter and T7 RNA polymerase, showing inclusion of hairpin eiRNA sequences.

Figure 2 is a graph showing HBsAg inhibition corresponding to data found in Table 2.

Figure 3 is a graph showing HBsAg inhibition corresponding to data found in Table 3.

Figure 4 is a graph showing HBsAg inhibition corresponding to data found in Table 4.

Figure 5 is a graph showing HBsAg inhibition corresponding to data found in Table 5.

Figure 6 is a graph showing HBsAg inhibition corresponding to data found in Table 6.

Figure 7 is a graph showing HBsAg inhibition corresponding to data found in Table 7.

Figure 8 is a graph showing HBsAg inhibition corresponding to data found in Table 8.

Figure 9 is a drawing depicting effective HBV-AYW shRNA inserts.

Figure 10 is a graph showing HBsAg inhibition corresponding to data found in Table 9.

Figure 11 is a bar graph showing downregulation of HBV RNA by Northern Blot analysis.

Figure 12 is a graph showing showing HBsAg inhibition corresponding to data found in Table 12.

Figure 13 is a Western Blot showing levels of HCV NS5A protein at (l to r) 0, 9, and 20 pmole of the identified siRNAs, as described in more detail in Experiment 1 of Example 2.

Figure 14 is a Western Blot showing levels of HCV NS5A protein at (l to r) 0, 9, and 20 pmole of the identified siRNA, and 0, 3, and 9 pmole of the "core" positive control siRNA, as described in more detail in Experiment 2 of Example 2.

DETAILED DESCRIPTION OF THE INVENTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing or transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Since RNA interference acts in a sequence specific manner, the RNAi molecule used as a drug must be specific to its target. Viral genomes are variable to accommodate resistance to changes in the environment. While HBV and HCV are very desirable viral targets for RNAi, the variability and mutability of the viruses

and the high rates of transcription of the viruses make HBV and HCV very challenging targets for any therapeutic and/or prophylactic approach. In order to knock down viral genome replication using RNAi there is a need to identify conserved and unique regions in the viral genome. At that same time, it is very important in order to avoid toxicity that any sequences selected for gene silencing be absent from the human genome.

Human Hepatitis B Virus (HBV) Hepatitis B virus belongs to the family of hepadnaviruses. The HBV genome is a relaxed circular, partially double stranded DNA of approximately 3,200 base pairs. There are 4 partially overlapping open reading frames encoding the envelope (pre-S/S), core (precore/core), polymerase, and X proteins. The pre-S/S open reading frames encode the large (L), middle (M), and small (S) surface glycoproteins. The precore/core open reading frame is translated into a precore polypeptide, which is modified into a soluble protein, the hepatitis B e antigen (HBeAg) and the nucleocapsid protein, hepatitis B core antigen. Mutations in the core promoter and precore region have been shown to decrease or abolish HBeAg production. The polymerase protein functions as a reverse transcriptase as well as a DNA polymerase. The X protein is a potent transactivator and may play a role in hepatocarcinogenesis.

The replication cycle of HBV begins with the attachment of the virion to the hepatocyte. Inside the hepatocyte nucleus, synthesis of the plus strand HBV DNA is completed and the viral genome is converted into a covalently closed circular DNA (cccDNA). Most antiviral agents that have been examined so far have little or no effect on cccDNA, which accounts for the rapid reappearance of serum HBV DNA after cessation of antiviral therapy. The aims of treatment of chronic hepatitis B are to achieve sustained suppression of HBV replication and/or expression of HBV antigens and remission of liver disease.

In GenBank version 132.0 there are more then 4500 HBV sequences and 340 HBV complete genome sequences (317 Human isolates, 22 isolates from other primates and one woodchuck HBV isolate).

This variability constitutes a serious challenge for sequence-specific pharmaceutical approaches such as RNAi. In order to identify conserved sequences suitable for RNAi applications, a comparison between all the complete genomes was carried out using a modified version of ClustalW. Two multiple alignment schemes were generated: the first included all 339 HBV complete genome sequences and the second was limited to all Human HBV isolates. The multiple alignment results were parsed and a table that included scores for sequence conservation at each position in the HBV genome was generated. A sliding window search to identify the longest region of sequence conservation larger then 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank accession no.: AF090840, a Human HBV isolate. The conserved HBV sequences were screened against Genbank sequences of both human genomic and cDNA libraries (Human chromosomes database). It was found that the identified conserved viral sequences are unique from 21 nt up. For human therapeutic purposes, assuring that homologous human sequences are not inadvertently silenced is as important as selecting conserved viral sequences for RNAi.

Human Hepatitis C Virus HCV is a small (40 to 60 nanometers in diameter), enveloped, single-stranded RNA virus of the family Flaviviridae and genus hepacivirus. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids, which is post-transcriptionally cleaved into 10 polypeptides, including 3 major structural (C, E1, and E2) and multiple non-structural proteins ([NS] NS2 to NS5). The NS proteins include enzymes necessary for protein processing (proteases) and viral replication (RNA polymerase). Because the virus mutates rapidly, changes in the envelope proteins may help it evade the immune system. There are at least 6 major genotypes and more than 90 subtypes of HCV. The different genotypes have different geographic distributions. Genotypes 1a and 1b are the most common in the United States (about 75 % of cases). Genotypes 2a and 2b (approximately 15%) and 3 (approximately 7%) are less common.

There is little difference in the severity of disease or outcome of patients infected with different genotypes. However, patients with genotypes 2 and 3 are more likely to respond to interferon treatment. The virus replicates at a high rate in the liver and has marked sequence heterogeneity. The main goal of treatment of chronic hepatitis C is to eliminate detectable viral RNA from the blood. Lack of detectable hepatitis C virus RNA from blood six months after completing therapy is known as a sustained response. Studies suggest that a sustained response is equated with a very favorable prognosis and that it may be equivalent to a cure. There may be other more subtle benefits of treatment, such as slowing the progression of liver scarring (fibrosis) in patients who do not achieve a sustained response.

In GenBank version 134.0 there are more then 20,000 HCV sequences and 93 HCV complete genome sequences. A comparison between all the complete genomes was carried out using a modified version of ClustalW. The multiple alignment result was parsed and a table that included scores for sequence conservation at each position in the HCV genome was generated. A sliding window search to identify the longest region of sequence conservation larger then 19 nt in length was created. Three major conserved regions were identified and mapped to GenBank RefSeq (reference sequence) accession no.: NC_004102 this is GenBank annotated HCV complete genome. The conserved sequences were screened against Genbank sequences of both human genomic and cDNA libraries (human chromosomes database), and those sequences unique from 21 nt up were identified.

Non-Homology with Human Sequences

It is equally important to ensure that conserved viral sequences targeted for silencing according to the invention be substantially non-homologous to any naturally occurring, normally functioning, and essential human polynucleotide sequence, so that the dsRNA molecule does not adversely affect the function of any essential naturally occurring

mammalian polynucleotide sequence, when used in the methods of this invention. Such naturally occurring functional mammalian polynucleotide sequences include mammalian sequences that encode desired proteins, as well as mammalian sequences that are non-coding, but that provide for essential regulatory sequences in a healthy mammal. Essentially, the RNA molecule useful in this invention must be sufficiently distinct in sequence from any mammalian polynucleotide sequence for which the function is intended to be undisturbed after any of the methods of this invention are performed. Computer algorithms may be used to define the essential lack of homology between the RNA molecule polynucleotide sequence and the normal mammalian sequences.

Since the length of a contiguous dsRNA sequence capable of association with and activation of RISC (RNA-induced silencing complex), is generally considered to be 19-27 base pairs, the identified conserved HBV and HCV sequences were compared with both human genomic libraries and, perhaps even more importantly, with human cDNA libraries. Since human cDNA libraries represent expressed sequences that appear in mRNAs, such mRNA sequences would be especially vulnerable to silencing by homologous dsRNA sequences provided to a cell.

Accordingly, the conserved HBV and HCV sequences were compared with human genomic and cDNA sequences. No human cDNA library matches to the HBV or HCV conserved sequences were identified. (Although there were some matches that were ultimately identified as HBV contamination in the cDNA library.) A comparison with human genomic library sequences revealed no match of any sequence of 21 nts or more, one match of 20 nucleotides, and one match of 19 nucleotides. These matches were in non-coding regions, and likely do not appear in mRNA since cognates were not turned up in the cDNA library. Therefore, they are considered unlikely to be a safety risk, but could be excluded if desired.

Conserved sequences from HBV and HCV

HBV Conserved Region 1

GAACATGGAGA[A(89%)/G(11%)]CA[T(76%)/C(24%)][C(78%)/A(20%)/T(2%)][A(78%)/G(21%)/T(1%)]CATCAGGA[T(65%)/c(35%)]TCCTAGGACCCCTGCTCGTGTTACAGGCGG[G(88%)/t(12%)]GT[T(89%)/G(11%)]TTTCT[T(94%)/C(6%)]GTTGACAA[G(64%)/A(36%)]AATCCTCACAATACC[A(56%)/G(43%)/T(1%)]CAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGG[G(92%)/A(5%)/T(3%)]A[A(41%)/G(30%)/T(18%)/C(11%)][C(90%)/T(10%)]

HBV Conserved Region 2

TGGATGTGTCT[G(99%)/A(1%)]CGGCGTTTTATCAT

HBV Conserved Region 3

AAGGCCTTTCT[A(43%)/G(43%)/C(14%)][T(56%)/A(37%)/C(7%)]GT[A(87%)/C(13%)]AACA[A(57%)/G(43%)]TA[T(59%)/C(41%)][C(59%)/A(41%)]TG[A(92%)/C(8%)][A(93%)/C(7%)]CCTTTACCCCGTTGC[T(54%)/C(46%)][C(92%)/A(8%)]GGCAACGG[C(74%)/T(24%)]C[A(50%)/T(43%)/c(7%)]GG[T(87%)/C(13%)]CT[G(70%)/C(19%)/T(7%)/A(4%)]TGCCAAGTGTTTGCTGACGCAACCCCCACTGG[C(48%)/T(38%)/A(14%)]TGGGGCTTGG[C(84%)/T(16%)][C(84%)/T(12%)/G(4%)]AT[A(47%)/T(23%)/G(17%)/C(13%)]GGCCATC[A(83%)/G(17%)][G(92%)/C(8%)]CGCATGCGTGGAACCTTT[G(84%)/C(13%)/T(3%)][T(92%)/A(4%)/C(3%)/G(1%)]G[G(78%)/T(22%)]CTCCTCTGCCGATCCATACTGCGGAACTCCT[A(88%)/T(9%)/G(1%)/C(1%)]GC[C(57%)/A(35%)/T(6%)/G(2%)]GC[T(92%)/C(7%)/G(1%)]TGTTT[T(88%)/C(12%)]GCTCGCAGC[C(64%)/A(36%)]GGTCTGG[A(87%)/G(13%)]GC

HBV Conserved Region 4

[C(62%)/T(38%)]ACTGTTCAAGCCTCAAGCTGTGCCTTGGGTGGCTTT[G(88%)/A(12%)]GG[G(92%)/A(8%)]CATGGACATTGAC[C(92%)/A(8%)]C[T(65%)/G(35%)]TATAAAGAATTTGGAGCT[A(65%)/T(35%)]

CTGTGGAGTTACTCTC[G(62%)/T(35%)/A(3%)]TTTTTGCCTTC[T(92%)/C(8%)]GACTT[C(92%)/T(8%)]TTTCCTTC

HBV Conserved Region 5

[C(69%)/del(31%)][G(69%)/del(31%)]A[G(85%)/T(11%)/C(4%)]GCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACG[C(61%)/A(39%)]G[A(62%)/G(38%)]TCTCAATCG[C(88%)/A(12%)]CGCGTCGCAGAAGATCTCAAT[C(92%)/T(8%)]TCGGGAATCT[C(88%)/T(12%)]AATGTTAGTAT

HBV Conserved Region 6

TTGG[C(84%)/t(16%)][C(84%)/t(12%)/g(4%)]AT[A(47%)/t(23%)/g(17%)/c(13%)]GGCCATC[A(83%)/g(17%)][G(92%)/c(8%)]CGCATGCGTGGAACCTTT[G(84%)/c(13%)/t(3%)][T(92%)/a(4%)/c(3%)/g(1%)]G[G(78%)/t(22%)]CTCCTCTGCCGATCCATACTGCGGAACTCCT[A(88%)/t(9%)/g(1%)/c(1%)]GC[C(57%)/a(35%)/t(6%)/g(2%)]GC[T(92%)/c(7%)/g(1%)]TGTTT[T(88%)/c(12%)]GCTCGCAGC[C(64%)/a(36%)]GGTCTGG[A(87%)/g(13%)]GC

HBV Conserved Region 7

CTGCCAACTGGAT[C(86%)/T(10%)/A(4%)]CT[C(69%)/T(25%)/A(6%)]CGCGGGACGTCCTTTGT[T(75%)/C(25%)]TACGTCCCGTC[G(93%)/A(7%)]GCGCTGAATCC[C(86%)/T(7%)/A(7%)]GCGGACGACCC[C(52%)/G(25%)/T(19%)/A(4%)]

HCV Conserved Region 1

[A(74%)/G(19%)/T(7%)][G(82%)/A(15%)/T(3%)]ATCACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGT[C(92%)/T(7%)]GTGCAGC[C(89%)/T(10%)]TCCAGG[A(76%)/T(14%)/C(8%)/G(1%)]CCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCC[A(90%)/G(9%)]GGA[C(78%)/T(16%)/A(5%)]GACCGGGTCCTTTCTTGGAT[G(78%)/T(11%)/A(10

%)]AACCCGCTC[A(94%)/T(5%)]ATGCC[T(90%)/C(9%)]GGA[G(91%)/C(4%)/A(4%)]ATTTGGGCGTGCCCCCGC[G(85%)/A(14%)]AGAC[T(94%)/C(5%)]GCTAGCCGAGTAG[T(92%)/C(7%)]GTTGGGT[C(94%)/T(5%)]GCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA[C(62%)/T(30%)/A(8%)]CATGAGCAC[A(50%)/G(50%)][A(92%)/C(8%)][A(89%)/T(11%)]TCC[T(92%)/A(5%)/C(3%)]AAACC[T(84%)/C(14%)/A(2%)]CAAAGAAAAACCAAA[C(84%)/A(16%)]G[T(84%)/A(16%)]AACACCAACCG[C(77%)/T(23%)]CGCCCACAGGACGT[C(81%)/T(18%)/A(1%)]AAGTTCCCGGG[C(89%)/T(11%)]GG[T(80%)/C(20%)]GG[T(80%)/C(17%)/A(3%)]CAGATCGTTGG[T(91%)/C(8%)/G(1%)]GGAGT[T(87%)/A(11%)/C(2%)]TAC[C(74%)/T(20%)/G(6%)]TGTTGCCGCGCAGGGGCCC[C(87%)/T(8%)/A(4%)/G(1%)][A(92%)/C(8%)][G(92%)/A(5%)/C(2%)][G(87%)/A(12%)/T(1%)]TTGGGTGTGCGCGCGAC[T(78%)/G(13%)/A(7%)/C(2%)]AGGAAGACTTC[C(90%)/G(5%)/T(5%)]GA[G(90%)/A(10%)]CGGTC[G(79%)/C(12%)/A(8%)/T(1%)]CA[A(86%)/G(14%)]CC[T(88%)/A(6%)C(6%)]CG[T(82%)/C(9%)A(9%)]GG[A(87%)/T(8%)/G(3%)/C(2%)]AG

HCV Conserved Region 2

ATGGC[T(76%)/A(12%)/C(10%)/G(2%)]TGGGATATGATGATGAACTGG[T(81%)/C(19%)]C

Conserved Consensus Sequences presented in SEQ ID format

The following sequences are presented in the format required per the WIPO Standard ST.25 (1998), using the codes provided under 37 CFR 1.821. SEQ ID NO:1 through SEQ ID NO:10 are derived from the HBV genome SEQ ID NO:11 and SEQ ID NO:12 are derived from the HCV genome.

SEQ ID NO:1 HBV

GAACATGGAGArCAyhdCATCAGGAyTCCTAGGACCCCTGCTCGTGTTACAGGCGGkGTkTTTCTyGTTGACAArAATCCTCACAATA

CCdCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGdA
ny

SEQ ID NO:2 HBV
TGGATGTGTCTrCGGCGTTTTATCAT

SEQ ID NO:3 HBV
AAGGCCTTTCTvhGTmAACArTAymTGmmCCTTTACCCC
GTTGCymGGCAACGGyChGGyCTnTGCCAAGTGTTTGCTGACGCAAC
CCCCACTGGhTGGGGCTTGGybATnGGCCATCrsCGCATGCGTGGAA
CCTTTbnGkCTCCTCTGCCGATCCATACTGCGGAACTCCTnGCnGCbT
GTTTyGCTCGCAGCmGGTCTGGrGC

SEQ ID NO:4 HBV
yACTGTTCAAGCCTCAAGCTGTGCCTTGGGTGGCTTTrG
GrCATGGACATTGACmCkTATAAAGAATTTGGAGCTwCTGTGGAGTTA
CTCTCdTTTTTGCCTTCyGACTTyTTTCCTTC

SEQ ID NO:5 HBV
CGAbGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCG
CAGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGA
ATCTyAATGTTAGTAT

SEQ ID NO:6 HBV
AbGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCA
GACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAAT
CTyAATGTTAGTAT

SEQ ID NO:7 HBV
CAbGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGC
AGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAA
TCTyAATGTTAGTAT

SEQ ID NO:8 HBV

GAbGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGC
AGACGmGrTCTCAATCGmCGCGTCGCAGAAGATCTCAATyTCGGGAA
TCTyAATGTTAGTAT

SEQ ID NO:9 HBV

TTGGybATnGGCCATCrsCGCATGCGTGGAACCTTTbnGk
CTCCTCTGCCGATCCATACTGCGGAACTCCTnGCnGCbTGTTTyGCTC
GCAGCmGGTCTGGrGC

SEQ ID NO:10 HBV

CTGCCAACTGGAThCThCGCGGGACGTCCTTTGTyTACG
TCCCGTCrGCGCTGAATCChGCGGACGACCCn

SEQ ID NO:11 HCV

DdATCACTCCCCTGTGAGGAACTACTGTCTTCACGCAGA
AAGCGTCTAGCCATGGCGTTAGTATGAGTGTyGTGCAGCyTCCAGGn
CCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTA
CACCGGAATTGCCrGGAhGACCGGGTCCTTTCTTGGATdAACCCGCT
CwATGCCyGGAvATTTGGGCGTGCCCCCGCrAGACyGCTAGCCGAGT
AGyGTTGGGTyGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTT
GCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCAhCATGAGCACrmw
TCChAAACChCAAAGAAAAACCAAAmGwAACACCAACCGyCGCCCAC
AGGACGThAAGTTCCCGGGyGGyGGhCAGATCGTTGGbGGAGThTAC
bTGTTGCCGCGCAGGGGCCCnmvdTTGGGTGTGCGCGCGACnAGGA
AGACTTCbGArCGGTCnCArCChCGhGGnAG

SEQ ID NO:12 HCV

ATGGCnTGGGATATGATGATGAACTGGyC

*Double Stranded RNA Gene Silencing/RNAi By "nucleic acid composition" or "nucleotide" composition is meant any one or more compounds in which one or more molecules of phosphoric acid are combined with a carbohydrate (e.g., pentose or hexose) which are in turn combined with bases derived from purine (e.g., adenine) and from pyrimidine (e.g., thymine). Particular naturally occurring nucleic acid molecules include genomic deoxyribonucleic acid (DNA) and host ribonucleic acid (RNA), as well as the several different forms of the latter, e.g., messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Also included are different DNA molecules which are complementary (cDNA) to the different RNA molecules. Synthesized DNA or a hybrid thereof with naturally occurring DNA, as well as DNA/RNA hybrids, and peptide nucleic acid (PNA) molecules (Gambari, Curr Pharm Des 2001 Nov;7(17):1839-62) can also be used.

It is contemplated that where the desired nucleic acid molecule is RNA, the T (thymine) in the sequences provided herein is substituted with U (uracil). For example, SEQ ID NO:1 through SEQ ID NO:44 are disclosed herein as DNA sequences. It will be obvious to one of ordinary skill in the art that an RNA effector molecule comprising sequences from any of the aforementioned SEQ ID NOs will have T substituted with U.

Nucleic acids typically have a sequence of two or more covalently bonded naturally-occurring or modified deoxyribonucleotides or ribonucleotides. Modified nucleic acids include, e.g., peptide nucleic acids and nucleotides with unnatural bases.

By "dsRNA" is meant a nucleic acid containing a region of two or more nucleotides that are in a double stranded conformation. It is envisioned that the conserved viral sequences of the invention may be utilized in any of the many compositions known in the art or subsequently developed which act through a dsRNA-mediated gene silencing or RNAi mechanism. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example,

by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. The dsRNA may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other. In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. Desirably, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary. Desirably, the region of the dsRNA that is present in a double stranded conformation includes at least 19, 20, 21, 22, 23, 24, 30, 50, 75,100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. Desirable RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (*e.g*, has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (*e.g*, has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid). In various embodiments, the RNA/DNA hybrid is made *in vitro* using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, a DNA strand synthesized *in vitro* is complexed with an RNA strand made *in vivo* or *in vitro* before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular

nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or a linear nucleic acid (see, for example, WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA *in vitro* or *in vivo* compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramide, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Patent No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed April 19, 2000, or U.S.S.N. 60/130,377, filed April 21, 1999. In other embodiments, the dsRNA contains coding sequence or non-coding sequence, for example, a regulatory sequence (e.g., a transcription factor binding site, a promoter, or a 5' or 3' untranslated region (UTR) of an mRNA). Additionally, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, filed April 19, 2000 (see, for example, pages 8-22), as well as any of the dsRNA molecules described in US Provisional Application 60/399,998 filed July 31, 2002, and PCT/US2003/024028, filed 31-Jul-2003; and US Provisional Application 60/419,532 filed October 18, 2002, and PCT/US2003/033466, filed 20-Oct-2003, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed *in vitro* or *in vivo* using the methods described herein or standard methods, such as those

described in WO 00/63364, filed April 19, 2000 (see, for example, pages 16-22).

dsRNA "Hairpin" Constructs: Constructs encoding a unimolecular hairpin dsRNA are more desirable for some applications than constructs encoding duplex dsRNA (i.e., dsRNA composed of one RNA molecule with a sense region and a separate RNA molecule with an antisense region) because the single-stranded RNA with inverted repeat sequences more efficiently forms a dsRNA hairpin structure. This greater efficiency is due in part to the occurrence of transcriptional interference arising in vectors containing converging promoters that generate duplex dsRNA. Transcriptional interference results in the incomplete synthesis of each RNA strand thereby reducing the number of complete sense and antisense strands that can base-pair with each other and form duplexes. Transcriptional interference can be overcome, if desired, through the use of (i) a two vector system in which one vector encodes the sense RNA and the second vector encodes the antisense RNA, (ii) a bicistronic vector in which the individual strands are encoded by the same plasmid but through the use of separate cistrons, or (iii) a single promoter vector that encodes a hairpin dsRNA, i.e., an RNA in which the sense and antisense sequences are encoded within the same RNA molecule. Hairpin-expressing vectors have some advantages relative to the duplex vectors. For example, in vectors that encode a duplex RNA, the RNA strands need to find and base-pair with their complementary counterparts soon after transcription. If this hybridization does not happen, the individual RNA strands diffuse away from the transcription template and the local concentration of sense strands with respect to antisense strands is decreased. This effect is greater for RNA that is transcribed intracellularly compared to RNA transcribed *in vitro* due to the lower levels of template per cell. Moreover, RNA folds by nearest neighbor rules, resulting in RNA molecules that are folded co-transcriptionally (i.e., folded as they are transcribed). Some percentage of completed RNA transcripts is therefore unavailable for base-pairing with a complementary second RNA because

of intra-molecular base-pairing in these molecules. The percentage of such unavailable molecules increases with time following their transcription. These molecules may never form a duplex because they are already in a stably folded structure. In a hairpin RNA, an RNA sequence is always in close physical proximity to its complementary RNA. Since RNA structure is not static, as the RNA transiently unfolds, its complementary sequence is immediately available and can participate in base-pairing because it is so close. Once formed, the hairpin structure is predicted to be more stable than the original non-hairpin structure. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002; and PCT/US2003/024028, "Double Stranded RNA Structures and Constructs and Methods for Generating and Using the Same," filed 31-Jul-2003; as well as the "udderly" structured hairpins, hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, "Double-Stranded RNA Structures and Constructs, and Methods for Generating and Using the Same," filed 20-Oct-2003.

By "short dsRNA" is meant a dsRNA that has about 50, 45, 40, 35, 30, 27, 25, 23, 21, 20 or 19 contiguous nucleotides in length that are in a double stranded conformation. Desirably, the short dsRNA is at least 19 basepairs in length. In desirable embodiments, the double stranded region is between 19 to 50, 19 to 40, 19 to 30, 19 to 25, 20 to 25, 21 to 23, 25 to 30, or 30 to 40 contiguous basepairs in length, inclusive. In some embodiments, the short dsRNA is between 30 to 50, 50 to 100, 100 to 200, 200 to 300, 400 to 500, 500 to 700, 700 to 1000, 1000 to 2000, or 2000 to 5000 nucleotides in length, inclusive and has a double stranded region that is between 38 and 60 contiguous basepairs in length, inclusive. In one embodiment, the short dsRNA is completely double stranded. In some embodiments, the short dsRNA is between 11 and 30 nucleotides in length, and the entire dsRNA is double stranded. In other embodiments,

the short dsRNA has one or two single stranded regions. In particular embodiments, the short dsRNA binds PKR or another protein in a dsRNA-mediated stress response pathway. Desirably, the short dsRNA inhibits the dimerization and activation of PKR by at least 20, 40, 60, 80, 90, or 100%. In some desirable embodiments, the short dsRNA inhibits the binding of a long dsRNA to PKR or another component of a dsRNA-mediated stress response pathway by at least 20, 40, 60, 80, 90, or 100%. See also the teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk, as to utilization of short dsRNAs in conjunction with other dsRNAs to avoid dsRNA-mediated toxicity.

By "at least 19 contiguous base pair nucleotide sequence" is meant that a nucleotide sequence can start at any nucleotide within one of the disclosed sequences, so long as the start site is capable of producing a polynucleotide of at least 19 base pairs. For example, an at least 19 contiguous base pair nucleotide sequence can comprise nucleotide 1 through nucleotide 19, nucleotide 2 through nucleotide 20, nucleotide 3 through nucleotide 21, and so forth to produce a 19mer. Thus, a 20mer can comprise nucleotide 1 through nucleotide 20, nucleotide 2 through nucleotide 21, nucleotide 3 through nucleotide 22, and so forth. Similar sequences above 20 contiguous nucleotides are envisioned.

By "expression vector" is meant any double stranded DNA or double stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or coding region of interest (*e.g.*, a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest, optionally, e.g., operatively linked to sequence lying outside a coding region, an antisense RNA coding region, a dsRNA coding region, or RNA sequences lying outside a coding region). Transfection or transformation of the expression vector into a recipient cell allows the cell to express RNA or protein encoded by the expression vector. An expression vector may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for

example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus.

By an "expression construct" is meant any double-stranded DNA or double-stranded RNA designed to transcribe an RNA, e.g., a construct that contains at least one promoter operably linked to a downstream gene or coding region of interest (*e.g.*, a cDNA or genomic DNA fragment that encodes a protein, or any RNA of interest). Transfection or transformation of the expression construct into a recipient cell allows the cell to express RNA or protein encoded by the expression construct. An expression construct may be a genetically engineered plasmid, virus, or artificial chromosome derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, or herpesvirus. An expression construct does not have to be replicable in a living cell, but may be made synthetically.

By "operably linked" is meant that a nucleic acid molecule and one or more regulatory sequences (e.g., a promoter) are connected in such a way as to permit transcription of the mRNA or permit expression and/or secretion of the product (i.e., a polypeptide) of the nucleic acid molecule when the appropriate molecules are bound to the regulatory sequences.

By a "promoter" is meant a nucleic acid sequence sufficient to direct transcription of a covalently linked nucleic acid molecule. Also included in this definition are those transcription control elements (*e.g.*, enhancers) that are sufficient to render promoter-dependent gene expression controllable in a cell type-specific, tissue-specific, or temporal-specific manner, or that are inducible by external signals or agents; such elements, which are well-known to skilled artisans, may be found in a 5' or 3' region of a gene or within an intron. Desirably a promoter is operably linked to a nucleic acid sequence, for example, a cDNA or a gene in such a way as to permit expression of the nucleic acid sequence.

The RNA molecule according to this invention may be delivered to the mammalian or extracellular pathogen present in the mammalian cell in the composition as an RNA molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made *in vitro* by conventional

enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1 57 Alternatively these molecules may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu et al., Nucleic Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin et al., Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby et al., Nucleic Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix et al., Nucleic Acids Res. 17:7381-93 (1989); S.H. Chou et al., Biochem., 28(6):2422-35 (Mar. 1989); 0. Odal el al., Nucleic Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin et al., Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun et al., RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang et al., Nucleic Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov el al., Nucleic Acids Res., 2-6 (18):4160-7 (Sept. 1998); M. Kadokura et al., Nucleic Acids Symp. Ser., 37:77-8 (1997); A. Davison et al., Biorned. Pept. Proteins. Nucleic Acids, 2(l):1-6 (1996); and A. V. Mudrakovskaia et al., Bioorg. Khirn., 17(6):819-22 (Jun. 1991)].

Still alternatively, the RNA molecule of this invention can be made in a recombinant microorganism, e.g., bacteria and yeast or in a recombinant host cell, e.g., mammalian cells, and isolated from the cultures thereof by conventional techniques. See, e.g., the techniques described in Sambrook et al, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, which is exemplary of laboratory manuals that detail these techniques, and the techniques described in US Patent Nos. 5.824,538; 5,877,159; and 5,643,771, incorporated herein by reference.

Such RNA molecules prepared or synthesized *in vitro* may be directly delivered to the mammalian cell or to the mammal as they are made *in vitro*. The references above provide one of skill in the art with the techniques necessary to produce any of the following specific embodiments, given the teachings provided herein. Therefore, in one

embodiment, the "agent" of the composition is a duplex (i.e., it is made up of two strands), either complete or partially double stranded RNA.

In another embodiment, the agent is a single stranded RNA sense strand. In another embodiment, the agent of the composition is a single stranded RNA anti-sense strand.

Preferably the single stranded RNA sense or anti-sense strand forms a hairpin at one or both termini. Desirably, the single stranded RNA sense or anti-sense strand forms a hairpin at some intermediate portion between the termini. Such a single stranded RNA sense or anti-sense strand may also be designed to fold back upon itself to become partially double stranded *in vitro* or *in vivo*. Yet another embodiment of an extant RNA molecule as the effective agent used in the compositions is a single stranded RNA sequence comprising both a sense polynucleotide sequence and an antisense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence. Preferably, this single stranded RNA sequence has the ability to become double-stranded once it is in the cell, or *in vitro* during its synthesis.

Still another embodiment of this invention is an RNA/DNA hybrid as described above.

Still another embodiment of the synthetic RNA molecule is a circular RNA molecule that optionally forms a rod structure [see, e.g., K-S. Wang et al., Nature 323:508-514 (1986)] or is partially double-stranded, and can be prepared according to the techniques described in S. Wang et al., Nucleic Acids Res., 22(12):2326-33 (June 1994); Y. Matsumoto et al., Proc. Natl. Acad. Sci, USA, 87(19):7628-32 (Oct. 1990); E. Ford & M. Ares, Proc. Natl. Acad. Sci. USA 91(8):3117-21 (Apr. 1994); M. Tsagris et al., Nucleic Acids Res., 19 7):1605-12 (Apr. 199l); S. Braun et al., Nucleic Acids Res. 24(21):4152-7 (Nov. 1996); Z. Pasman et al., RNA, 2(6):603-10 (Jun. 1996); P. G. Zaphiropoulos, Proc. Natl. Acad. Sci., USA, 93(13):6536-41 (Jun. 1996); D. Beaudry et al., Nucleic Acids Res., 23(15):3064-6 (Aug. 1995), all incorporated herein by reference. Still

another agent is a double-stranded molecule comprised of RNA and DNA present on separate strands, or interspersed on the same strand.

Alternatively, the RNA molecule may be formed *in vivo* and thus delivered by a "delivery agent" which generates such a partially double-stranded RNA molecule *in vivo* after delivery of the agent to the mammalian cell or to the mammal. Thus, the agent which forms the composition of this invention is, in one embodiment, a double stranded DNA molecule "encoding" one of the above-described RNA molecules. The DNA agent provides the nucleotide sequence which is transcribed within the cell to become a double stranded RNA. In another embodiment, the DNA sequence provides a deoxyribonucleotide sequence which within the cell is transcribed into the above-described single stranded RNA sense or anti-sense strand, which optionally forms a hairpin at one or both termini or folds back upon itself to become partially double stranded. The DNA molecule which is the delivery agent of the composition can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a nonbase paired polynucleotide sequence, and wherein the single stranded RNA sequence has the ability to become double-stranded. Alternatively, the DNA molecule which is the delivery agent provides for the transcription of the above-described circular RNA molecule that optionally forms a rod structure or partial double strand *in vivo*. The DNA molecule may also provide for the *in vivo* production of an RNA/DNA hybrid as described above, or a duplex containing one RNA strand and one DNA strand. These various DNA molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in the Promega reference, cited above.

A latter delivery agent of the present invention, which enables the formation in the mammalian cell of any of the above-described RNA molecules, can be a DNA single stranded or double stranded plasmid or vector. Expression vectors designed to produce RNAs as described herein *in vitro* or *in vivo* may contain sequences under the control of any RNA

polymerase, including mitochondrial RNA polymerase, RNA pol I, RNA pol II, and RNA po1 III, and viral polymerases, and bacteriophage polymerases such as T7 and Sp6. These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. Vectors may be desirably designed to utilize an endogenous mitochondrial RNA polymerase (e.g., human mitochondrial RNA polymerase, in which case such vectors may utilize the corresponding human mitochondrial promoter). Mitochondrial polymerases may be used to generate capped (through expression of a capping enzyme) or uncapped messages *in vivo*. RNA pol I, RNA pol II, and RNA pol III transcripts may also be generated *in vivo*. Such RNAs may be capped or not, and if desired, cytoplasmic capping may be accomplished by various means including use of a capping enzyme such as a vaccinia capping enzyme or an alphavirus capping enzyme. However, all pol II transcripts are capped. The DNA vector is designed to contain one of the promoters or multiple promoters in combination (mitochondrial, RNA pol I, pol II, or pol III, or viral, bacterial or bacteriophage promoters along with the cognate polymerases). Preferably, where the promoter is RNA pol II, the sequence encoding the RNA molecule has an open reading frame greater than about 300 nts and must follow the rules of design to prevent nonsense-mediated degradation in the nucleus. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages.

Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.

Thus, one exemplary vector is a single or double-stranded phage vector. Another exemplary vector is a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral

vectors may also be and preferably are introduced into cells as packaged or encapsidated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally occurs only in complementing host cells.

In another embodiment the delivery agent comprises more than a single DNA or RNA plasmid or vector. As one example, a first DNA plasmid can provide a single stranded RNA sense polynucleotide sequence as described above, and a second DNA plasmid can provide a single stranded RNA anti-sense polynucleotide sequence as described above, wherein the sense and anti-sense RNA sequences have the ability to base-pair and become double-stranded. Such plasmid(s) can comprise other conventional plasmid sequences, e.g., bacterial sequences such as the well-known sequences used to construct plasmids and vectors for recombinant expression of a protein. However, it is desirable that the sequences which enable protein expression, e.g., Kozak regions, etc., are not included in these plasmid structures.

The vectors designed to produce dsRNAs of the invention may desirably be designed to generate two or more, including a number of different dsRNAs homologous and complementary to a target sequence. This approach is desirable in that a single vector may produce many, independently operative dsRNAs rather than a single dsRNA molecule from a single transcription unit and by producing a multiplicity of different dsRNAs, it is possible to self select for optimum effectiveness. Various means may be employed to achieve this, including autocatalytic sequences as well as sequences for cleavage to create random and/or predetermined splice sites.

Other delivery agents for providing the information necessary for formation of the above-described desired RNA molecules in the mammalian cell include live, attenuated or killed, inactivated recombinant bacteria which are designed to contain the sequences necessary for the required RNA molecules of this invention. Such recombinant bacterial

cells, fungal cells and the like can be prepared by using conventional techniques such as described in US Patent Nos. 5,824,538; 5,877,159; and 5,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited reference, including, without limitation, Escherichia coli, Bacillus subtilis, Salmonella typhimurium, and various species of Pseudomonas, Streptomyces, and Staphylococcus.

Still other delivery agents for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required RNA polynucleotide sequence discussed above. Such viruses may be designed similarly to recombinant viruses presently used to deliver genes to cells for gene therapy and the like, but preferably do not have the ability to express a protein or functional fragment of a protein. Among useful viruses or viral sequences which may be manipulated to provide the required RNA molecule to the mammalian cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses, papova viruses (such as SV40), poliovirus, pseudorabies viruses, retroviruses, lentiviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viral delivery agents may be designed by applying conventional techniques such as described in M. Di Nocola et al., Cancer Gene Ther., 5(6):350-6 (1998), among others, with the teachings of the present invention.

The term "*in vivo*" is intended to include any system wherein the cellular DNA or RNA replication machinery is intact, including tissue culture systems, and within single cell or multicellular living organisms.

By "multiple sequitope dsRNA" or "multisequitope dsRNA" is meant an RNA molecule that has segments derived from multiple target nucleic acids or that has non-contiguous segments from the

same target nucleic acid. For example, the multiple sequitope dsRNA may have segments derived from (i) sequences representing multiple genes of a single organism; (ii) sequences representing one or more genes from a variety of different organisms; and/or (iii) sequences representing different regions of a particular gene (e.g., one or more sequences from a promoter and one or more sequences from an mRNA. Desirably, each segment has substantial sequence identity to the corresponding region of a target nucleic acid. In various desirable embodiments, a segment with substantial sequence identity to the target nucleic acid is at least 19, 20, 21, 22, 23, 24, 30, 40, 50, 100, 200, 500, 750, or more basepairs in length. In desirable embodiments, the multiple epitope dsRNA inhibits the expression of at least 2, 4, 6, 8, 10, 15, 20, or more target genes by at least 20, 40, 60, 80, 90, 95, or 100%. In some embodiments, the multiple epitope dsRNA has non-contiguous segments from the same target gene that may or may not be in the naturally occurring 5' to 3' order of the segments, and the dsRNA inhibits the expression of the nucleic acid by at least 50, 100, 200, 500, or 1000% more than a dsRNA with only one of the segments.

By "sequitope" is meant a contiguous sequence of double-stranded polyribonucleotides that can associate with and activate RISC (RNA-induced silencing complex), usually a contiguous sequence of between 19 and 27 basepairs, inclusive. Sequences comprising at least one sequitope from within one or more of the conserved HBV and/or HCV nucleotide sequences identified here may be utilized for dsRNA mediated gene silencing as taught herein.

Multiple-epitope dsRNAs The advantages of a multiple-epitope or multisequitope double-stranded RNA approach as taught in USSN 60/419,532, filed 18-Oct-2002 and PCT/US2003/033466, filed 20-Oct-2003, are applicable to utilization of the conserved HBV and/or HCV

sequences of the invention. Because a singular species of dsRNA can simultaneously silence many target genes (e.g., genes from multiple pathogens, multiple genes or sequences from a single pathogen, or genes associated with multiple diseases), a multiple epitope dsRNA can be used for many different indications in the same subject or used for a subset of indications in one subject and another subset of indications in another subject. For such applications, the ability to express long dsRNA molecules (e.g., dsRNA molecules with sequences from multiple genes) without invoking the dsRNA stress response is highly desirable. For example, by using a series of sequences, each, e.g., as short as 19-21 nucleotides, desirably 100 to 600 nucleotides, or easily up to 1, 2, 3, 4, 5, or more kilobases such that the total length of such sequences is within the maximum capacity of the selected plasmid (e.g., 20 kilobases in length), a single such pharmaceutical composition can provide protection against a large number of pathogens and/or toxins at a relatively low cost and low toxicity, e.g., HBV, HCV, HIV, etc.

The use of multiple epitopes derived from one or more genes from multiple strains and/or variants of a highly variable or rapidly mutating pathogen such as HBV and/or HCV can also be very advantageous. For example, a singular dsRNA species that recognizes and targets multiple strains and/or variants of HBV and/or HCV can be used as a universal treatment or vaccine for the various strains/variants of HBV and/or HCV.

The ability to silence multiple genes of a particular pathogen such as HBV and/or HCV prevents the selection of, in this case, HBV and/or HCV "escape mutants." In contrast, typical small molecule treatment or vaccine therapy that only targets one gene or protein results in the selection of pathogens that have sustained mutations in the target gene or protein and the pathogen thus becomes resistant to the therapy. By simultaneously targeting a number of genes or sequences of the pathogen and or extensive regions of the pathogen using the multiple epitope approach of the present invention, the emergence of such "escape mutants" is effectively precluded.

For example, it is considered particularly advantageous to include a mixture of sequences from both HCV SEQ ID NO:11 and SEQ ID NO:12, i.e., one or more sequences from HCV SEQ ID NO:11 together with one or more sequences from HCV SEQ ID NO:12, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient, in order to decrease the ability of the virus to generate viable escape mutants. Similarly, it would be advantageous to provide a mixture of the conserved HBV sequences, in some cases in combination with one or more of the conserved HCV sequences of the invention.

Similarly, it may be desirable to use sequences from two or more of HBV SEQ ID NO:1, SEQ ID NO:2, AND SEQ ID NO:3, either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient. SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 map to the HBV surface antigen genes. Due to the overlapping nature of the HBV mRNAs, the following mRNAs would be targeted by one of more of these sequences: Surface Ag (sAg) mRNAs, precore, core and polymerase mRNAs. However, since sAg mRNAs are the most abundant, it is more likely that these mRNAs will be targeted if the gene-silencing machinery is saturable. It is possible, however, that all listed mRNAs will be targeted. Reduction of surface Ag is desirable for several reasons: a) surface Ag is needed for assembly of infectious virions; b) overexpression of Surface Ag during infection is thought to contribute to immune anergy that occurs during chronic infection; and c) the expression of sAg in the livers of infected individuals (even in the absence of virus, i.e., from integrated sAg sequences into the host genome) induces hepatitis. Therefore, reduction of sAg is likely to decrease viral titers, overcome immune anergy and decrease/prevent hepatitis.

HBV SEQ ID NO:4 maps to the unique region of precore and core and will target these mRNAs specifically. Core protein is needed to make functional virions and so down regulation of this mRNA is predicted to

decrease viral titers. There should be no competition of these effector RNAs for surface, polymerase or X mRNAs.

HBV SEQ ID NO:5 through SEQ ID NO:8 map to the polymerase gene. Effector RNAs are predicted to target only precore/core and polymerase transcripts. There should be no competition with sAg or X mRNAs. Polymerase is needed for the synthesis of viral genomes and therefore viral particle titer is expected to decrease is polymerase is decreased.

HBV SEQ ID NO:9 maps to the X gene. Due to the terminal redundancy of all mRNAs, these effector RNAs have the potential to target all viral mRNAs. X protein has many ascribed (non proven) functions. However, the X gene is often found in integrated HBV sequences in individuals with active hepatitis and down-regulation of X gene expression is predicted to ameliorate disease.

In general, the more sequences from the different identified sequences that are used (e.g., from SEQ ID NO:1, SEQ ID NO:2, and/or SEQ ID NO:3, plus sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10), the less likely a virus will be able to generate viable escape mutants. Also, the more different mRNAs that can be targeted, the more significant will be the drops in viral titer and disease amelioration.

Desirable combinations for multiepitope constructs, an admixture of constructs, or the concomitant administration of different dsRNA constructs include the following: Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9; Sequences

from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus

sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 plus sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4 and SEQ ID NO:5; Sequences from SEQ ID NO:4 and SEQ ID NO:6; Sequences from SEQ ID NO:4 and SEQ ID NO:7; Sequences from SEQ ID NO:4 and SEQ ID NO:8; Sequences from SEQ ID NO:4 and SEQ ID NO:9; Sequences from SEQ ID NO:4 and SEQ ID NO:10; Sequences from SEQ ID NO:5 and SEQ ID NO:6; Sequences from SEQ ID NO:5 and SEQ ID NO:7; Sequences from SEQ ID NO:5 and SEQ ID NO:8; Sequences from SEQ ID NO:5 and SEQ ID NO:9; Sequences from SEQ ID NO:5 and SEQ ID NO:10; Sequences from SEQ ID NO:6 and SEQ ID NO:7; Sequences from SEQ ID NO:6 and SEQ ID NO:8; Sequences from SEQ ID NO:6 and SEQ ID NO:9; Sequences from SEQ ID NO:6 and SEQ ID NO:10; Sequences from SEQ ID NO:7 and SEQ ID NO:8; Sequences from SEQ ID NO:7 and SEQ ID NO:9; Sequences from SEQ ID NO:7 and SEQ ID NO:10; Sequences from SEQ ID NO:8 and SEQ ID NO:9; Sequences from SEQ ID NO:8 and SEQ ID NO:10; Sequences from SEQ ID NO:9 and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:6; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:8; and SEQ ID NO:10;

Sequences from SEQ ID NO:4, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:7;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:8;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:9;
Sequences from SEQ ID NO:5, SEQ ID NO:6; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:8;
Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:9;
Sequences from SEQ ID NO:5, SEQ ID NO:7; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:9;
Sequences from SEQ ID NO:5, SEQ ID NO:8; and SEQ ID NO:10;
Sequences from SEQ ID NO:5, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:8;
Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:9;
Sequences from SEQ ID NO:6, SEQ ID NO:7; and SEQ ID NO:10;
Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:9;
Sequences from SEQ ID NO:6, SEQ ID NO:8; and SEQ ID NO:10;
Sequences from SEQ ID NO:6, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:7, SEQ ID NO:8; and SEQ ID NO:9;
Sequences from SEQ ID NO:7, SEQ ID NO:8; and SEQ ID NO:10;
Sequences from SEQ ID NO:7, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:8, SEQ ID NO:9; and SEQ ID NO:10;
Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:6; and SEQ ID NO:7; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:6; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5; SEQ ID NO:9; and

SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6; SEQ ID NO:7; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:8; and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7; SEQ ID NO:9; and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID

NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; Sequences from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; and Sequences from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10.

In an another embodiment, combinations of sequitopes and longer sequences from within any of the aforementioned sequences (e.g., SEQ ID NO:1 through SEQ ID NO:12) may be utilized either in a single dsRNA construct, an admixture of constructs, or through concomitant administration of such constructs to a patient.

As discussed elsewhere herein, a particularly preferred embodiment of the invention utilizes dsRNA expression constructs or vectors to achieve endogenous delivery of the dsRNAs of the invention, especially the multiple different sequences described above. These dsRNAs may be provided e.g., on the same cistron of an expression construct such as a plasmid, on different cistrons of an expression construct, or on different expression constructs or plasmids, e.g., one or more plasmids and/or one or more vectors, including viral vectors. Such multiple different sequences may also be provided exogenously, in any different mixture of one or more dsRNA structures, duplexes and/or harpins, and/or in combination with one or more endogenously expressed dsRNA structures.

Desirable methods of administration of nucleic acids The DNA and/or RNA constructs of the invention may be administered to the host cell/tissue/organism as "naked" DNA, RNA, or DNA/RNA, formulated in a pharmaceutical vehicle without any transfection promoting agent. More efficient delivery may be achieved as known to those of skill in the art of DNA and RNA delivery, using e.g., such polynucleotide transfection facilitating agents known to those of skill in the art of RNA and/or DNA delivery. The following are exemplary agents: cationic amphiphiles including local anesthetics such as bupivacaine, cationic lipids, liposomes or lipidic particles, polycations such as polylysine, branched, three-dimensional polycations such as dendrimers, carbohydrates, detergents, or surfactants, including benzylammonium surfactants such as benzalkonium chloride. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U.S. Patent numbers 5,593,972; 5,703,055; 5,739,118; 5,837,533; 5,962,482; 6,127,170; 6,379,965; and 6,482,804; and International Patent Application No. PCT/US98/22841; the teaching of which is hereby incorporated by reference. U.S. Patents numbers 5,824,538; 5,643,771; and 5,877,159 (incorporated herein by reference) teach delivery of a composition other

than a polynucleotide composition, e.g., a transfected donor cell or a bacterium containing the dsRNA-encoding compositions of the invention.

In some embodiments, the dsRNA or dsRNA expression vector is complexed with one or more cationic lipids or cationic amphiphiles, such as the compositions disclosed in US 4,897,355 (Eppstein *et al.*, filed October 29, 1987), US 5,264,618 (Felgner *et al.*, filed April 16, 1991) or US 5,459,127 (Felgner *et al.*, filed September 16, 1993). In other embodiments, the dsRNA or dsRNA expression vector is complexed with a liposome/liposomic composition that includes a cationic lipid and optionally includes another component such as a neutral lipid (see, for example, US 5,279,833 (Rose), US 5,283,185 (Epand), and US 5,932,241).

Particularly desirable methods and compositions for delivery of the oligonucleotide compositions of the invention for pharmaceutical applications, including for targeted delivery to hepatocytes, are described in PCT/US03/14288, filed May 6, 2003, the teaching of which is incorporated herein by reference.

Transformation/transfection of the cell for research and other non-therapeutic purposes may occur through a variety of means including, but not limited to, lipofection, DEAE-dextran-mediated transfection, microinjection, calcium phosphate precipitation, viral or retroviral delivery, electroporation, or biolistic transformation. The RNA or RNA expression vector (DNA) may be naked RNA or DNA or local anesthetic complexed RNA or DNA (*See* U.S. Patent Nos. 6,217,900 and 6,383,512, "Vesicular Complexes and Methods of Making and Using the Same, Pachuk *et al.*, *supra*).

Another desirable delivery technology for the dsRNAs or dsRNA expression constructs of the invention for pharmaceutical applications is the self-assembling Cyclosert™ two-component nucleic acid delivery system, based on cyclodextrin-containing polycations, which are available from Insert Therapeutics, Pasadena, CA. (See Bioconjug Chem 2003 May-Jun; 14 (3): 672-8; Popielarski et al.; "Structural effects of

carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization"; as well as Bioconjug Chem 2003 Jan-Feb;14 (1):247-54 and 255-61.) The first component is a linear, cyclodextrin-containing polycationic polymer, that when mixed with DNA, binds to the phosphate "backbone" of the nucleic acid, condensing the DNA and self assembling into uniform, colloidal nanoparticles that protect the DNA from nuclease degradation in serum. A second component is a surface modifying agent with a terminal adamantine-PEG molecule, that when combined with the cyclodextrin polymer forms an inclusion complex with surface cyclodextrins and prevents aggregation, enhances stability and enables systemic administration. In addition, targeting ligands to cell surface receptors may be attached to the modifier providing for targeted delivery of DNA directly to target cells of interest. Since hepatocytes are susceptible to HBV and HCV infection, utilizing this method to target delivery of the dsRNA expression constructs of the invention to liver cells is considered especially advantageous. E.g., the asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes may be targeted by use of synthetic ligands with galactosylated or lactosylated residues, such as galactosylated polymers.

In general, targeting for selective delivery of the dsRNA constructs of the invention to hepatocytes is.preferred. Targeting to hepatocytes may be achieved by coupling to ligands for hepatocyte-specific receptors. For example, asialo-orosomucoid, (poly)L-lysine-asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess, Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992; Wu et al., Biotherapy 3:87-95, 1991). Similarly, the oligonucleotides may be targeted to hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte-specific receptors. Oligonucleotides may also be targeted to hepatocytes using specific vectors, as described below.

Particularly preferred compositions for delivery of dsRNAs or dsRNA expression constructs of the invention are the multifunctional

compositions as described in PCT/US03/14288, filed May 6, 2003, which may include trilactosyl spermine as a ligand for targeting to the ASG Receptor of hepatocytes. Trilactosyl cholesteryl spermine co-complexes with the oligonucleotides of the invention may be prepared and used as described to transfect hepatocytes *in vivo*.

The dsRNA oligonucleotides of the invention may be provided exogenously to a target hepatocyte. Alternatively, a dsRNA may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding the dsRNA. In this method, the nucleic acid molecule is contained within a non-replicating linear or circular DNA or RNA molecule, or is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfect a hepatocyte may be used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO89/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993; Graham et al., in Murray, ed., Methods in Molecular Biology: Gene Transfer and Expression Protocols. Vol. 7, Clifton, N.J.: the Human Press 1991: 109-128), adeno-associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sep. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and any modified versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Appropriate regulatory sequences can be inserted into the vectors of the invention using methods known to those skilled in the art, for example, by homologous recombination (Graham et al., J. Gen. Virol. 36:59-72, 1977), or other appropriate methods (Molecular Cloning: A

Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, N.Y., 1989).

Promoters Promoters are inserted into the vectors so that they are operably linked, typically but not invariably, 5' to the nucleic acid sequence encoding the dsRNA oligonucleotide. Any promoter that is capable of directing initiation of transcription in a eukaryotic cell may be used in the invention. For example, non-tissue-specific promoters, such as the cytomegalovirus (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein), mouse metallothionine I gene (Hammer, et al., J. Mol. Appl. Gen. 1:273-288, 1982), HSV thymidine kinase (McKnight, Cell 31:355-365 1982), and SV40 early (Benoist et al., Nature 290:304-310, 1981) promoters may be used. Non-tissue-specific promoters may be used in the invention, as expression of HBV and/or HCV dsRNA in non-liver cells directed by the non-tissue-specific promoters should be harmless to the non-liver cells, because of the specificity of the HBV and HCV dsRNAs of the invention for viral sequences. However, preferred promoters for use in the invention are hepatocyte-specific promoters, the use of which ensures that the RNAs are expressed primarily in hepatocytes. Preferred hepatocyte-specific promoters include, but are not limited to the albumin, alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from cytomegalovirus, herpes simplex viruses (types I and II), hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention.

dsRNA expression vectors may include promoters for RNA polymerase I, RNA polymerase II including but not limited to HCMV, SCMV, MCMV, RSV, EF2a, TK and other HSV promoters such as ICP6, ICP4 and ICP0 promoters, HBV pregenomic promoter, RNA pol III promoter including but not limited to U6 and tRNA promoters, mitochondrial light and heavy strand promoters. Desirably, the dsRNA expression vector comprises at least one RNA polymerase II promoter, for

example, a human CMV-immediate early promoter (HCMV-IE) or a simian CMV (SCMV) promoter, at least one RNA polymerase I promoter, or at least one RNA polymerase III promoter. The promoter may also be a T7 promoter, in which case, the cell further comprises T7 RNA polymerase. Alternatively, the promoter may be an SP6 promoter, in which case, the cell further comprises SP6 RNA polymerase. The promoter may also be one convergent T7 promoter and one convergent SP6 RNA promoter. A cell may be made to contain T7 or SP6 polymerase by transforming the cell with a T7 polymerase or an SP6 polymerase expression plasmid, respectively. In some embodiments, a T7 promoter or a RNA polymerase III promoter is operably linked to a nucleic acid that encodes a short dsRNA (e.g., a dsRNA that is less than 200, 150, 100, 75, 50, or 25 basepairs in length). In other embodiments, the promoter is a mitochondrial promoter that allows cytoplasmic transcription of the nucleic acid in the vector (see, for example, the mitochondrial promoters described in WO 00/63364, filed April 19, 2000, and in WO/US2002/00543, filed 9-Jan-2001). Alternatively, the promoter is an inducible promoter, such as a *lac* (Cronin et al. Genes & Development 15: 1506-1517, 2001), *ara* (Khlebnikov et al., J Bacteriol. 2000 Dec;182(24):7029-34), ecdysone (Rheogene website), RU48 (mefepristone) (corticosteroid antagonist) (Wang XJ, Liefer KM, Tsai S, O'Malley BW, Roop DR, Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8483-8), or *tet* promoter (Rendal et al., Hum Gene Ther. 2002;13(2):335-42 and Larnartina et al., Hum Gene Ther. 2002;13(2):199-210) or a promoter disclosed in WO 00/63364, filed April 19, 2000. Also useful in the methods and compositions of the invention are the structural and chimeric promoters taught in USSN 60/464,434, filed 22-Apr-2003. See also the promoter systems taught in Pachuk, C., and Satishchandran, C., "Multiple-Compartment Eurkaryotic Expression Systems," U.S. Provisional Application No. 60/497,304, filed 22-Aug-2003, which are considered particularly desirable in the methods and compositions of the invention.

Liver specific promoters useful in dsRNA expression constructs of the invention include the albumin promoter, the alpha-fetoprotein promoter (especially in liver cancer cells), the alpha-1-antitrypsin promoter, hepatitis B promoters, e.g., hepatitis B promoters including promoters for the antigen genes, including core, e antigen, polymerase and X protein.

T7 Promoter/T7 Polymerase Expression Systems A desirable method of the invention utilizes a T7 dsRNA expression system to achieve cytoplasmic expression of dsRNA, (e.g., long or short dsRNA molecules) in vertebrate cells (e.g., mammalian cells). The T7 expression system utilizes the T7 promoter to express the desired dsRNA. Transcription is driven by the T7 RNA polymerase, which can be provided on a second plasmid or on the same plasmid. Bacteriophage T7 RNA polymerase (T7 Pol) is the product of T7 gene 1, which can recognize its responsive promoter sequence specifically and exhibit a high transcriptase activity. The complete sequence of the T7 genome, with detailed information about the different regions of the bacteriophage, including promoter sequences, is disclosed in Dunn & Studier, 1983, J. Mol. Biol. 166(4), 477-535 (see also NCBI 'Genome' database, Accession No. NC 00 1 604). The T7 promoter cannot be utilised by any other RNA polymerase than the polymerase of bacteriophage T7, which shows a stringent specificity for the promoter (Chamberlin et al., 1970, Nature 228:227-231). When utilizing the T7 expression system for expressing dsRNAs, for example, a first plasmid construct that expresses both a sense and antisense strand under the control of converging T7 promoters and a second plasmid construct that expresses the T7 RNA polymerase under the control of an RSV promoter can be used. Both the dsRNA and the T7 RNA polymerase could advantageously be expressed from a single bicistronic plasmid construct, particularly when the dsRNA is formed from a single RNA strand with inverted repeats or regions of self-complementarity that enable the strand to assume a stem-loop or hairpin structure with an at least partially double stranded region. Individual sense and antisense strands which self assemble to form a dsRNA can be synthesized by a single plasmid

construct using, e.g., converging promoters such as bacteriophage T7 promoters placed respectively at the 5' and 3' ends of the complementary strands of a selected sequence to be transcribed. See also, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 60/419,532, filed 18-Oct-2002.

Therapeutic Compositions of the Invention The dsRNAs of the invention, and the recombinant vectors containing nucleic acid sequences encoding them, may be used in therapeutic compositions for preventing or treating HCV and/or HBV infection. The therapeutic compositions of the invention may be used alone or in admixture, or in chemical combination, with one or more materials, including other antiviral agents. Currently, lamivudine, adefovir dipivoxil, and interferon alpha have been approved for treatment of HBV, and it is anticipated that the compositions of the invention may be used in combination with these and other drugs that are active against HBV, including emtricitabine (FTC) and entecavir. Since dsRNAs against HBV and/or HCV act through a novel mechanism (dsRNA-mediated gene silencing/RNAi), combination therapy of the agents of the invention and other antivirals is expected to significantly increase the efficacy of therapy while substantially reducing the development of drug resistance, e.g., the development of lamivudine resistance, a problem of major concern with long term lamivudine therapy. Currently, interferon and ribavirin are licensed for treatment of HCV, and as for HBV, it is anticipated that the compositions of the invention may be used in combination with these and other drugs that are active against HCV. Specific dosage regimens involving therapy with such multiple agents can be determined through routine experimentation by those of ordinary skill in the art of clinical medicine.

Formulations will desirably include materials that increase the biological stability of the oligonucleotides or the recombinant vectors, or materials that increase the ability of the therapeutic compositions to penetrate hepatocytes selectively. The therapeutic compositions of the

invention may be administered in pharmaceutically acceptable carriers (e.g., physiological saline), which are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. One having ordinary skill in the art can readily formulate a pharmaceutical composition that comprises an oligonucleotide or a gene construct. In some cases, an isotonic formulation is used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in Remington: The Science and Practice of Pharmacy (formerly Remington's Pharmaceutical Sciences), Mack Publishing Co., a standard reference text in this field, and in the USP/NF.

Routes of administration include, but are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraoccularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intravenous, intramuscular, oral, intraperitoneal, intradermal, intraarterial and subcutaneous injection. dsRNAs or dsRNA expression constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Alternatively, the dsRNA and/or dsRNA expression construct may be introduced by various means into cells that are removed from the individual. Such means include, for example, *ex vivo* transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated therein can be implanted into the

individual even if the host cells were originally taken from another individual.

In HBV infected individuals it is anticipated that the dsRNA compositions of the invention may be useful as a pre-treatment in conjunction with therapeutic vaccination protocols designed to boost immunity against the virus. It is also anticipated that the dsRNA compositions of the invention may be useful for prophylaxis in a regimen of periodic administrations to individuals who because of occupational or other potential for exposure are considered at high risk of exposure to HBV and/or HCV, e.g., fire, emergency, and health care personnel. Such an effective prophylactic regime may include administration of a composition that provides an HBV and/or HCV dsRNA of the invention, e.g., weekly, biweekly, monthly, bimonthly, every three months, every four months, semi-yearly, or yearly, as can be determined through routine experimentation by those of skill in the art of clinical medicine. The ability of a dsRNA expression vector such as a plasmid or viral vector to express the dsRNAs of the invention over a relatively prolonged period of time, expected to be in the range of weeks to months, is considered to be advantageous for this and other applications.

Dosage of dsRNAs For administration of dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 5 µg to 500 µg dsRNA is administered to a 90-150 pound person/animal (in order of increasing preference). For administration of a vector encoding dsRNA (e.g., a short dsRNA to inhibit toxicity or a short or long dsRNA to silence a gene) to an animal, typically between 100 mg to 300 mg, 10 mg to 100 mg, 1 mg to 10 mg, 500 µg to 1 mg, or 50 µg to 500 µg dsRNA expression vector or construct is administered to a 90-150 pound person/animal (in order of increasing preference). The dose may be adjusted based on the weight of the animal. In some embodiments, about 1 to 10 mg/kg or about 2 to 2.5 mg/kg is administered. Other doses

may also be used, as determined through routine experimentation by those of skill in the art of clinical medicine.

For administration in an intact animal, typically between 10 ng and 50 µg, between 50 ng and 100 ng, or between 100 ng and 5 µg of dsRNA or DNA encoding a dsRNA is used. In desirable embodiments, approximately 10 µg of a DNA or 5 µg of dsRNA is administered to the animal. With respect to the methods of the invention, it is not intended that the administration of dsRNA or DNA encoding dsRNA to cells or animals be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration sufficient to provide a dose adequate to inhibit gene expression, prevent a disease, or treat a disease.

If desired, short dsRNA is delivered before, during, or after the exogenous delivery of dsRNA (e.g., a longer dsRNA) that might otherwise be expected to induce cytotoxicity. See the teaching of USSN 10/425,006, filed 28-Apr-2003, "Methods of Silencing Genes Without Inducing Toxicity", Pachuk.

Applicants specifically incorporate the entire content of all cited references in this disclosure. Further, when an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

EXAMPLES

The following Examples are provided as illustrative only. All references mentioned within this disclosure are specifically incorporated herein by reference in their entirety.

EXAMPLE 1

Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model: A human liver-derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession V01460. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

Using this model, cells were co-transfected with the infectious molecular clone of HBV and various eiRNA constructs. The cells were then monitored for loss of HBV expression and replication as described below. Details on the vector and encoded RNAs used in this experiment are provided at the end of this example.

Experiment 1:

The following is an example of an experiment that was performed using eiRNA vectors encoding sequences derived from GenBank accession number V01460. HBV sequences in these described eiRNA vectors were highly conserved sequences identified as described elsewhere herein, which also exhibited activity as siRNAs (See, Pachuk, C., "Methods and Constructs for Evaluation of RNAi targets and Effector Molecules," PCT/US2004/005065, filed 25-Feb-2004). The particular eiRNA backbone vector used for this experiment was a proprietary vector containing a U6 promoter to drive expression of the encoded RNAs. Each vector encoded only one short hairpin RNA (shRNA). The shRNA coding sequence was followed by an RNA pol III termination sequence. Sequences of the U6 promoter, RNA pol III termination signal, and encoded shRNAs are all shown at the end of the example. Similar vectors containing U6 promoters and RNA pol III termination signals are commercially available such as the "siLentGene-2 Cloning Systems" vector from Promega, Inc., Madison, Wis. One of ordinary skill in the art can also create them according to the information provided herein. It is expected that similar results would also be obtained using other expression and promoter systems especially those vectors with RNA pol III promoters that are not U6, for example H1 promoters or 7SK promoters.

Experimental Procedure: Transfection.

Huh7 cells cultured in RPMI-1640 media were seeded into six-well plates at a density of $3x10^5$ cells/well. All transfections were performed the day after cell seeding using Lipofectamine™ (InVitrogen, Carlsbad, Cal.) according to the manufacturer's directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype ("pHBV2") (GenBank Accession # V01460) and 500 ng, 300ng, 250ng, 120 ng, 100 ng, 50ng, or 10 ng of an eiRNA construct. DNA was held constant/transfection at 2.5 µg by including an inert plasmid DNA, pGL3-Basic (Promega, Madison Wis.) in amounts that brought the total DNA in

the transfection to 2.5 µg. For example, in transfections receiving 500 ng of HBV DNA and 500 ng of an eiRNA construct, 1.5 µg pGL3 was added to the transfection. Prior to transfection, media was removed from the cells and the cells washed with Opti-MEM® (InVitrogen Life Technologies, Carlsbad, Cal.). 800 µl of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® were removed from cells and replaced with 2 mL culture media/well. At 3, 6, and 10 days after transfection, the media was removed from cells and stored at –70 °C. The media was replaced with 2 mL of fresh culture media on days 3 and 6. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 µg pGL3) and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 1 µg control eiRNA construct, and 1.0 µg pGL3 DNA).

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at days 3, 6, and 10 post-transfection. The Auszyme® ELISA, commercially available from Abbott Labs (Abbott Park, Ill.), was used to detect surface Ag (sAg) according to the manufacturer's instructions. sAg was measured since surface Ag is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone and from HBVcccDNA produced during infection *in vivo*. Since surface Ag synthesis can continue with deleterious effects in the absence of HBV replication, it is important to down-regulate not only viral replication but also replication-independent synthesis of sAg.

Results:

Cells transfected with the HBV-specific eiRNA constructs described at the end of this example all induced a decrease in sAg levels relative to the controls. The level inhibition is shown in the accompanying FIG. 2-8 corresponding to data found in Tables 2-8. Note that the sequences identified as 788-808 and 807-827 only lowered surface Ag levels by 30% and 50% respectively at 500 ng doses. These are the only two eiRNAs that do not target the sAg mRNA; instead they target the 3.1 Kb HBV mRNAs and therefore reduce sAg levels indirectly. The 30% to 50% reduction in sAg observed when these other HBV RNAs are targeted is considered a strong indication that these eiRNA constructs are efficacious.

HBV-specific eiRNAs used in this experiment

The eiRNA vectors encode the HBV sequences listed in Table 1. The sequences are shown as well as their map coordinates on GenBank accession number V01460. At the rightmost part of the table is the SEQ ID NO that these sequences map within. The sequence of the encoded RNA is 5'GGTCGAC (a sequence that is *per se* unimportant, but is derived from the polylinker sequence of the particular vector used) followed by a first sense or antisense HBV sequence followed by the loop sequence (underlined in Table 1) followed by a second HBV sequence, which is the complement to the first HBV sequence. Note that the loop structure does not need to be a fixed sequence or length, and we have used several loop sequences with no significant impact on the functioning of the eiRNA construct. The second HBV sequence is followed by a string of T residues, e.g., 1, 2, 3, or more Ts, that function as the termination signal for RNA pol III.

Table 1

HBV-AYW coordinates*	SEQ ID NO	sequence (sense stem - loop - antisense stem)	Maps within SEQ ID NO
788-808	14	CGTCTGCGAGGCGAGGGAGTTAGAGAACTTAACTCCCTCGCCTCGCAGACG	5, 6, 7, or 8
807-827	15	TTCTTCTTCTAGGGGACCTGCAGAGAACTTGCAGGTCCCCTAGAAGAAGAA	5, 6, 7, or 8
1291-1311	16	AAGCCACCCAAGGCACAGCTTAGAGAACTTAAGCTGTGCCTTGGGTGGCTT	4
1299-1319	17	CAAGGCACAGCTTGGAGGCTTAGAGAACTTAAGCCTCCAAGCTGTGCCTTG	4
1737-1757	18	GGATTCAGCGCCGACGGGACGAGAGAACTTCGTCCCGTCGGCGCTGAATCC	10
1907-1927	19	TTCCGCAGTATGGATCGGCAGAGAGAACTTCTGCCGATCCATACTGCGGAA	3
1912-1932	20	CAGTATGGATCGGCAGAGGAGAGAGAACTTCTCCTCTGCCGATCCATACTG	3
1943-1963	21	TCCACGCATGCGCTGATGGCCAGAGAACTTGGCCATCAGCGCATGCGTGGA	3
1991-2011	22	TGCGTCAGCAAACACTTGGCAAGAGAACTTTGCCAAGTGTTTGCTGACGCA	3
2791-2811	23	AAAACGCCGCAGACACATCCAAGAGAACTTTGGATGTGTCTGCGGCGTTTT	2
2791-2811mut	24	AAAACACCACACACGCATCCAAGAGAACTTTGGATGCGTGTGTGGTGTTTT	2
2912-2932	25	TTGAGAGAAGTCCACCACGAGAGAGAACTTCTCGTGGTGGACTTCTCTCAA	1
2919-2939	26	AAGTCCACCACGAGTCTAGACAGAGAACTTGTCTAGACTCGTGGTGGACTT	1

*nucleotide coordinates refer to Genbank accession number V01460.

A diagram of the transcribed RNA structure is shown in **FIG. 9.**

SEQ ID NO:13 is the nucleotide sequence of U6 promoter. Nucleotide sequence of RNA pol III terminator: 5' – TTTTT – 3'.

Tables and Graphs.

HBsAg was measured as described above and plotted in **FIG. 2-8** corresponding to the data in Tables 2-8. The amount of eiRNA construct is shown in parentheses following the name of the eiRNA construct and is in µg amounts. For example, 2791(0.5) means that 0.5 µg or 500 ng of eiRNA construct 2791-2811 (see Table 1) was used in the transfection. The percent inhibition relative to the control is also shown in the tables below and it is specific for the day 10 measurement. Note that the 4th set of data in this example in which 1299 was evaluated at 500 ng has only two timepoints, days 3 and 6, because the evaluation was not carried out at day 10. The percent inhibition for this experiment was shown for day 6 data. Data is shown as raw OD data collected as described by the manufacturer of the Auszyme ELISA assay kit used to measure sAg. Not shown are the 50 ng data for 2791-2811 and the 10ng data for 1907-1927. Each of these doses inhibited HBsAg expression by about 50% relative to the control.

Table 2

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.339	1.88	3.268	——
2791(0.5)	0.101	0.263	0.333	89.8

Table 3

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	1.169	4.445	10.18	-----
2791(0.5)	0.442	0.743	1.3	87.2
2791Mut(0.5)	1.136	4.305	10.595	-----

Table 4

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.375	1.952	4.005	-----
2791mut(1)	0.421	1.847	4.753	-----
HCV(1)	0.445	1.805	3.933	-----
788(0.5)	0.255	1.195	2.778	30.6
807(0.5)	0.254	1.326	2.015	49.7
1907(0.25)	0.052	0.113	0.365	90.9
1912(0.25)	0.138	0.208	0.517	87.1
1943(0.25)	0.099	0.233	0.506	87.4
1991(0.25)	0.075	0.152	0.291	92.7
2912(0.25)	0.095	0.183	0.331	91.7

Table 5

	Day 3	Day 6	% Inhibition relative to control
pHBV2	0.474	1.513	-----
1299(0.5)	0.439	0.699	53.8

Table 6

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.33	1.617	2.88	-----
2791(0.3)	0.103	0.192	0.349	87.9
1737(0.3)	0.051	0.094	0.232	91.9
1291(0.12)	0.239	0.587	1.195	58.5
1907(0.12)	0.043	0.086	0.356	87.6
2919(0.12)	0.218	0.565	1.09	62.2

Table 7

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.741	2.53	5.383	-----
2791(0.3)	0.223	0.256	0.458	91.5
1737(0.1)	0.212	0.351	0.549	89.8
1907(0.1)	0.067	0.149	0.468	91.3
1991(0.1)	0.067	0.16	0.345	93.6

Table 8

	Day 3	Day 6	Day 10	% Inhibition relative to control
pHBV2	0.864	4.414	8.344	-----
1907(0.05)	0.17	0.538	1.396	83.3
2919(0.1)	0.368	1.044	1.908	77.1
1291(0.2)	0.573	1.654	1.896	77.3

Experiment 2:

Background: The same cell culture model was used to evaluate the additive effects of adding two eiRNA constructs. In this experiment 2791-2811 and 2919-2939 were evaluated. They were evaluated separately at two doses: 10 ng and 25 ng, and in combination at 10 ng (5 ng of 2791-2811 plus 5 ng of 2919-2939) and at 25 ng (12.5 ng 2791-2811 plus 12.5ng 2919-2939). An additive effect is observed, for example, when half the inhibition seen with 25 ng 2791-2811 plus half the inhibition seen with 25 ng 2919-2939 is about equal to the inhibition seen of the 25 ng combination dose. This is important because while one may not be gaining inhibition over the use of a single eiRNA construct at the 25 ng dose, the use of two or more eiRNA sequences is very important in preventing the generation of viral escape mutants.

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates at a density of $3x10^5$ cells/well. All transfections were performed the day after cell seeding using Lipofectamine™ (InVitrogen) according to the manufacturer's

directions. In this experiment, cells were transfected with 500 ng of the infectious HBV plasmid ayw subtype (GenBank Accession # V01460) and 25 ng or 10 ng of two separate eiRNA constructs or a combination of these two eiRNA constructs at a total of 25 ng or 10 ng. DNA was held constant/transfection at 2.5 µg by including an inert plasmid DNA, pGL3, in amounts that brought the total DNA in the transfection to 2.5 µg. For example, in transfections receiving 500 ng of HBV DNA and 10 ng of an eiRNA construct, then 1.99 µg pLUC was added to the transfection. Prior to transfection, media was removed from the cells and the cells washed with Opti-MEM® (InVitrogen Life Technologies). 800 µl of Opti-MEM® was then added to each well of cells followed by the addition of the transfection mix. Seventeen to nineteen hours post-transfection, the transfection mix and Opti-MEM® was removed from cells and replaced with 2 mL culture media/well. At 4, 8, and 11 days after transfection, the media was removed from cells and stored at –70 °C. The media was replaced with 2 mL of fresh culture media on days 4 and 8. All transfections were carried out in duplicate. Two sets of control transfections were also performed: HBV DNA alone (500 ng HBV DNA plus 2 µg pGL3), and HBV DNA with a control eiRNA construct (500 ng HBV DNA, 500 ng control eiRNA construct and 1.5µg pGL3. DNA).

Results:

Results are shown in Table 9, and the corresponding graph found in **FIG. 10**. Combining 2791-2811 and 2919-2939 showed at least equal effects to administration of 2791-2811 or 2919-2939 alone. It is expected that similar advantages will be seen by combining two or more dsRNAs directed to different HBV sequences from the same and/or different HBV genes.

Table 9

	Day 4	Day 8
$pHBV_2$	3.74	15.03
2791 @ 25 ng	2.49	9.63
2919 @ 25 ng	2.55	10.07
2791 + 2919 @ 25 ng	2.73	10.91

Experiments 3 and 4

Silencing of HBV in a mouse model.

Summary: Two of the eiRNA vectors described in confirmatory experiment 1 were assessed for their ability to silence an HBV replicon in a mouse model. These vectors were the 2791-2811 and the 1907-1927 vectors. Both vectors were found to silence HBV in the mouse model to a similar extent as they silenced in the cell culture model. The ability to silence this HBV replicon in mice by other therapeutics has been demonstrated to be a predictor of human efficacy [4].

Animal Model Background:

Chimpanzees represent the only animal model in which to study human HBV infectivity. A mouse model is available, however, in which HBV expression and replication occur. This model has been invaluable for the evaluation of anti-HBV therapeutic agents not only targeted against viral replication but also against RT-independent expression of antigen. In this model, replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic delivery [1].

The aim of the following experiment was to test two of the vectors encoding HBV-specific sequences evaluated in Experiment 1 for efficacy in a mouse model even though there were not expected to be HBV-sequence-related efficiency differences between the cell culture and mouse models. This experiment utilized hydrodynamic delivery as a method to co-deliver replication competent HBVayw plasmid (Example 1,

confirmatory experiment 1) with an effector HBV-specific eiRNA expression vector. Hydrodynamic delivery is ideal for these first studies because it results in efficient delivery of nucleic acid to the liver [5].

Experiments.

Hydrodynamic Delivery Studies: Experiment 3.

All animals were hydrodynamically injected with 7.5 µg infectious HBVayw plasmid (described in confirmatory Example 1). Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals. Experimental animals were co-injected with 10 µg 2791-2811. A second group of control animals were injected with 10 µg of an irrelevant eiRNA construct. All animals were also co-injected with 2.5 µg of a GFP reporter plasmid (Clontech, Palo Alto, Cal.). Expression of GFP mRNA in the livers of injected mice served as a control to normalize results against the mouse model transfection efficiency. Total DNA injected in animals was kept at a constant 20 µg by including pGL3, an inert filler DNA (Promega, Madison, Wis.). All DNA was formulated and injected according to the methods described in Yang et al. [1]. There were 5 animals per group. The DNAs and amounts of DNA injected per animal are shown in Table 10.

Table 10

Group	HBV DNA	GFP DNA	eiRNA	pGL3
1	7.5 µg	2.5 µg	10 µg 2791	0 µg
2	7.5 µg	2.5 µg	10 µg control	0 µg
3	7.5 µg	2.5 µg	0 µg	10 µg

Timepoints of analysis were selected based on published results from Dr. Chisari's laboratory [1], which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery. Serum was assayed for the presence of HBsAg on days 1, 2, 3, and 4 post-injection. Assays were performed as described for the cell culture model of HBV replication. The presence of HBV RNA in liver samples was ascertained by Northern blot analysis on day 2 following injection using procedures developed in Dr. Chisari's laboratory [1] and normalized to endogenous GAPDH RNA levels and GFP mRNA levels using conventional techniques, or a quantitative RT-PCR assay for HBV RNAs containing sAg coding sequences using standard techniques. RT-PCR is more quantitative than Northern Blot analysis and has a larger dynamic window than does Northern Blot analysis.

Downregulation of both HBV RNA by Northern Blot analysis and HBsAg were seen in mice injected with 2791-2811. See **FIG. 11**. Also not shown, quantitative RT-PCR demonstrated the presence of 867 HBV RNA molecules in the livers of control mice and 57 molecules of HBV RNA in 2791-2811 treated mice, a 15-fold downregulation.

Table 11

Group	Mouse	3.5 kb	2.1 kb	GFP	HBV total	HBV/GFP	Group Average	Std. Dev
10 µg 2791	1	182360	1440614	4044344	1622974	4.0	3.0	0.76
	2	392294	3161703	9954889	3553997	3.6		
	3	268673	3114347	15317275	3383020	2.2		
	4	394799	3909096	16806285	4303895	2.6		
	5	362182	4439430	18306755	4801612	2.6		
HBV only	21	2412562	8720964	3860082	11133526	28.8	17.4	7.40
	22	2170741	7958388	6110744	10129129	16.6		
	23	2713213	12060855	9633404	14774068	15.3		
	24	1924373	7243024	11042915	9167397	8.3		
	25	1464641	5726217	3968243	7190858	18.1		

Table 12

NUC5_HBsAg		HBsAg (ng/ml)			
		d1	d2	d3	d4
HBV	2	2810	6793	8422	8517
	3	2344	8332	8089	8743
	4	1684	8788	9064	8876
	5	2318	9378	8597	8480
	29	1066	5038	5153	5925
	grp ave=>	2044	7666	7865	8108
	Std Dev=>	678	1754	1556	1231
eiHCV	6	2554	8048	9233	8870
	9	2267	8420	9535	8338
	10	1704	8258	8761	7840
	30	1362	4171	5406	4920
	grp ave=>	1972	7224	8234	7492
	Std Dev=>	538	2041	1912	1765
2791	11	1262	2823	2276	2080
	12	1222	2549	2858	1593
	14	1056	1933	1143	792
	15	1275	8320	1920	2068
	27	779	4771	3782	1252
	grp ave=>	1119	4079	2396	1557
	Std Dev=>	209	2598	993	551

Hydrodynamic Delivery Studies: Experiment 4.

This experiment was similar to the Experiment 3 of Example 1 except that two eiRNA constructs were evaluated: 2791-2811 and 1907-1927. In this experiment, HBsAg was measured on days 1 and 4 using the assay already described herein.

Table 13

NUC6_HBsAg		HBsAg (ng/ml)	
		d1	d4
HBV	2	6147 ^	36,953 ^
	3	6234 ^	42,542 ^
	4	4658	33,061 ^
	5	5077 ^	29,389 ^
	grp ave=>	5529	35486
	Std Dev=>	784	5627
eiHCV	6	1901	11,236
	7	6286 ^	29,637 ^
	8	1023	6,345
	grp ave=>	3070	15739
	Std Dev=>	2820	12282
2791	11	3966	5009
	13	4705	7347 ^
	14	2289	4538
	15	2427	4217
	grp ave=>	3347	5278
	Std Dev=>	1182	1417
1907	16	4954	7203 ^
	18	2982	6917 ^
	19	3436	7568 ^
	20	2246	5135 ^
	grp ave=>	3405	6706
	Std Dev=>	1143	1081

EXAMPLE 2

Hepatitis C- Sequences for RNAi Therapeutic Development

Experiment 1

Brief Introduction:

The hepatitis C virus (HCV) is the primary cause of non-A, non-B transfusion-associated hepatitis and accounts for more than 200 million hepatitis cases worldwide. The HCV genome has a high degree of sequence variability. There are six major genotypes comprising more than

fifty subtypes and significant heterogeneity hallmarked by quasi-species has been found within patients. Great progress in understanding HCV replication has been made by using recombinant polymerases or cell-based subgenomic replicon systems. By using a replicon cell system, HCV-specific siRNA has been demonstrated to be able to suppress HCV protein expression and RNA replication. Sequences of the 5' NTR and both structural and nonstructural genes have been targeted successfully. The highly conserved nature of the 3' NTR sequence makes it a highly attractive target for siRNA based therapy. However, no study has been done to examine the feasibility of using the 3' NTR. Here we report the design and testing of several siRNAs that can inhibit HCV protein expression in the subgenomic replicon system. Exogenously synthesized HCV-specific siRNAs were transfected into the HCV replicon cell line as described below.

Cell culture and media:

The HCV replicon in hepatoma Huh7 cells was cultured in Dulbecco's Modified Eagle Media ("DMEM") (Invitrogen) containing 10% fetal calf serum (Invitrogen), 1% penicillin-streptomycin, 1% non-essential amino acids and 0.5 mg/mL Geneticin. Cells were grown to 75% confluency prior to splitting.

Western blot analysis:

Total cell lysates from replicon cells were harvested from replicon cells in 1× LDS Buffer (Invitrogen). The lysates were heated at 90 °C for 5 min in the presence of beta-mercaptoethanol before electrophoresis on a 10% Tris-Glycine polyacrylamide gel (Invitrogen). The protein was transferred to PVDF (Invitrogen) membrane. Following the transfer, the membrane was rinsed once with PBS containing 0.5% Tween-20 (PBS-Tween) and blocked in PBS-Tween containing 5% non-fat milk for 1 hr. After washing with PBS-Tween, the membrane was incubated with the

primary α-NS5A antibody (a gift from Dr. Chen Liu) at 1:1500 dilution for 1 hr at room temperature. Prior to incubation with HRP conjugated α-mouse IgG secondary antibody (Amersham) diluted 1:5000, the blot was washed in PBS-Tween 20. Following the secondary antibody incubation, the blot was washed again and treated with ECL (Amersham) according to the manufacturer's protocol.

Northern blot:

Total cellular RNA was extracted by using the Rneasy® kit (Qiagen). Northern blot analysis was done according to the protocol of Guo et al. Briefly, 5 µg total RNA was electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and immobilized by UV cross-linking (Stratagene). Hybridization was carried out using α-[^{32}P]CTP-labeled neomycin RNA in a solution containing 50% deionized formamide, 5× SSC (750 mM sodium chloride, 750 mM sodium citrate), Denhardt's solution, 0.02 M sodium phosphate (pH 6.8), 0.2% sodium dodecyl sulfate ("SDS"), 100 µg of sheared denatured salmon sperm DNA/ml, and 100 µg of yeast RNA/ml, for 16 hr at 58 °C. The membranes were washed once in 2× SSC/0.1% SDS for 30 min at room temperature and twice in 0.1× SSC/0.1% SDS for 30 min at 68 °C. Membranes were exposed to X-ray film.

Transfection of siRNA into replicon cells:

For transfection of siRNA into replicon cells the Lipofectamine® 2000 reagent (Invitrogen) was used according to the user manual. Briefly, $2x10^4$ cells in 0.5 mL of DMEM was seeded in 24 well plates one day before the transfection. The indicated amount of siRNA was diluted in 50 µL OptiMEM and mixed with diluted Lipofectamine® 2000 reagent (1 µL in 50 µL of Optimem). The mixture was incubated at room temperature for 20 min before being applied onto the cell monolayer. 48-72 hr after

transfection, cells were washed in PBS and lysed in 100 µL SDS sample buffer.

Table 14

siRNA number	SEQ ID NO	HCV sequence
#12	28	GCTAAACACTCCAGGCCAATACCTGTCTC
#22	29	TCCTTTGGTGGCTCCATCTTACCTGTCTC
#32	30	GCTCCATCTTAGCCCTAGTCACCTGTCTC
#42	31	TCTTAGCCCTAGTCACGGCTACCTGTCTC
#52	32	CCTAGTCACGGCTAGCTGTGACCTGTCTC
#62	33	CTAGTCACGGCTAGCTGTGAACCTGTCTC
#72	34	CGTGAGCCGCTTGACTGCAGACCTGTCTC
#82	35	GCTGATACTGGCCTCTCTGCACCTGTCTC
#102	36	ACTGGCCTCTCTGCAGATCAACCTGTCTC

Several siRNAs comprising the HCV sequences identified above in Table 14 targeting the 3'UTR; siRNA #12 targeting the HCV NS5B gene (positive control); the identified HCV core siRNA (positive control); and the identified lamin siRNA (negative control) were synthesized using the Silencer siRNA construction kit, Catalog # 1620 (Ambion Inc., Austin, Tex.). DNA oligonucleotides were synthesized by IDT (Coralville, Iowa).

Control siRNAs:

1. HCV core (positive control): SEQ ID NO:45
2. #12, shown in Table 14, targeting the HCV NS5B gene, also a positive control
3. lamin sequence (negative control): SEQ ID NO:46

Three siRNAs were used as controls: siRNA targeting the cellular gene Lamin for negative control; siRNA targeting the core sequence of HCV as a positive control; siRNA targeting the HCV NS5B gene as a positive control. Two concentrations of each siRNA (9 and 20 pmole) were used and the results were compared with transfection of no siRNA.

Accordingly, the Western Blots in **FIG. 13** represent 0, 9, and 20 pmoles of the identified siRNAs. siRNA #22, 32, 42, 62, and 72 were notably active in repressing HCV NS5A protein expression. Presumably, HCV RNA level is also decreased based on the results obtained previously with positive control siRNA for core. Several siRNAs had minimum effect at the concentrations tested and should be evaluated at higher concentrations. These include #12 (targeting NS5B), #102, #52, and #82.

Experiment 2

Experiment 2 was performed as described in Experiment 1 of Hepatitis C-Sequences for RNAi Therapeutic Development except that siRNAs R1-R8, comprising the sequences (and their complements) set forth in Table 15 below, were used in transfections. The Western Blot assay performed here was as described in Example 2, Experiment 1. The control HCV core siRNA used as a positive control is the siRNA described in the previous HCV Experiment 1. All siRNAs were transfected at concentrations of 0, 9, and 20 pmole except the control "core" siRNA, which was transfected at levels of 0, 3, and 9 pmole. R1, R2, R3, R5, R7, and R8 all exhibited significant inhibition of HCV as can be seen in the Western Blot, **FIG. 14.**

Table 15

siRNA	SEQ ID NO	HCV sequence
R1	37	CTGGCCTCTCTGCAGATCAAG
R2	38	TGCAGAGAGTGCTGATACTGG
R3	39	TGAGCCGCTTGACTGCAGAGA
R4	40	GAAAGGTCCGTGAGCCGCTT
R5	41	TAGCTGTGAAAGGTCCGTGAG
R6	42	TTAGCCCTAGTCACGGCTAGC
R7	43	TCCATCTTAGCCCTAGTCACG
R8	44	TTGGTGGCTCCATCTTAGCCC

All siRNAs evaluated map to the 3'UTR of the HCV genome and are conserved amongst HCV genotypes and quasi-species. SEQ ID NO:27 represents this 101 nt sequence of the HCV 3'UTR, sometimes referred to as the "X" region.

EXAMPLE 3

Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model:

A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession #s V01460 and J02203. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirrors HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg (Hepatitis B Surface Antigen) particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells are co-transfected with the infectious molecular clone of HBV and the individual effector RNA constructs to be evaluated. The cells are then monitored for loss of HBV expression and replication as described below.

The following is an example of an experiment using eiRNA vectors encoding sequences derived from SEQ ID NO:1 and SEQ ID NO:5. The particular eiRNA vectors for this experiment are T7 RNA polymerase-

based (See, e.g., the teaching of WO 0063364, with respect to T7 dsRNA expression systems, as well as USSN 60/399,998P, filed 31-Jul-2002 and USSN 60/419,532, filed 18-Oct-2002) and encode hairpin RNA structures (especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the "udderly" structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003). It is expected that similar results will be obtained using other expression and promoter systems, e.g., as described above, and/or vectors encoding alternative dsRNA structures (i.e. duplex).

Experimental Procedure: Transfection.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluency at the time of transfection. All transfections are performed using Lipofectamine™ (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50 ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid below) 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below) and 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:5 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections to bring total DNA/transfection up to 2.5 µg DNA.

Monitoring cells for loss of HBV expression.

Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface antigen (HBsAg). HBsAg is measured since HBsAg is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface antigen cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6]. Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is then purified by phenol/chloroform extraction and ethanol precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

Results will desirably indicate a 70-95% decrease in both HBsAg and viral particle secretion in the media of cells transfected with the HBV plasmid, T7 RNA polymerase expression plasmid and eiRNA constructs relative to cells transfected with only the HBV plasmid and T7 RNA polymerase expression plasmid.

Vectors used in experiment

Sequence of the T7 RNA polymerase gene

SEQ ID NO:47 represents the T7 RNA polymerase gene which is cloned into a mammalian expression vector such as pCEP4 (Invitrogen, Carlsbad,CA). Cloning can be easily done by one skilled in the art. One skilled in the art would also be aware that a leader sequence with a Kozak sequence needs to be cloned in directly upstream from the T7 RNA polymerase gene.

eiRNA vector encoding RNA hairpin derived from SEQ ID NO:1

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (about 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

eiRNA vector encoding RNA hairpin derived from SEQ ID NO:5

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 730-786 of GenBank accession #s V01460 and J02203. The hairpin is designed as described for hairpin encoding sequences from SEQ ID NO:1.

EXPERIMENT 1

Rationale for mouse models:

Chimpanzees represent the only animal model in which to study human HBV infectivity. Mouse models are available, however, in which human HBV expression and replication occur. These models have been invaluable for the evaluation of anti-HBV therapeutic agents and have been shown to be a predictor for the efficacy of these agents in humans

[4]. The first of these models are transgenic mouse models, in which the HBV genome or selected HBV genes are expressed [7,8]. Because HBV is integrated into the mouse genome, these animals serve as a model not only for viral replication but also for RT-independent expression of antigen. A similar model exists in which replication competent HBV is expressed transiently from episomal HBV DNA. This model is created by introducing replication competent HBV DNA into mouse liver by hydrodynamic delivery [1]. Unlike the transgenic animals, these mice are not immunotolerant to HBV antigens and immune-mediated clearance of HBV transfected hepatocytes can be studied.

Although woodchuck and duck models exist for the study of woodchuck hepatitis (WHBV) and duck hepatitis (DHBV) respectively, we have opted not to use these models for several reasons. 1) Human HBV cannot be studied in these models. As we are ultimately interested in down-regulating expression of human HBV, use of these models would at some point necessitate the re-design and evaluation of vectors and/or RNAs specific for human HBV. 2) the mice are isogenic and therefore noise due to genetic variables within the system does not arise. 3) Unlike human HBV, there are no validated WHBV/DHBV cell culture models that can be studied in parallel with their respective animal models.

The experiment described below utilizes hydrodynamic delivery as a method to co-deliver replication competent HBVayw plasmid with the various effector dsRNA (eiRNA) expression vectors. Hydrodynamic delivery is ideal for this experiment because it results in efficient delivery of nucleic acid to the liver [5]. Combination of the dsRNA effector plasmid and replication competent HBV plasmid into the same formulation increases the likelihood that both plasmids are taken up by the same cells. Because expressed effector dsRNA are present in the majority of cells bearing the replicating HBV plasmid, observed results can be attributed to the performance of the effector plasmid rather than to differences in delivery efficiencies. This experiment demonstrates only that a particular eiRNA is efficacious in an infected liver. Formulation and delivery are not

addressed by this example. Formulation, dosing and delivery of the eiRNA vector are enabled in the example in which transgenic mice are used.

Experimental procedure:

Control B10.D2 mice are hydrodynamically injected with an infectious molecular clone of HBV (ayw subtype) consisting of a terminally redundant viral genome that is capable of transcribing all of the viral RNAs and producing infectious virus [1,2,3]. Following internalization into hepatocytes and nuclear localization, transcription of HBVayw plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication [1]. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the sera of injected animals. Animals are injected with four doses of the HBV replicon plasmid (1 µg, 3 µg, 5 µg, and 10 µg). These doses are chosen because they represent non-saturating doses capable of eliciting detectable expression of a reporter plasmid following hydrodynamic delivery. Animals are co-injected with the effector dsRNA expression vector (eiRNA) such that animals in each group receive a 10-19 µg dose of a particular effector construct(s) such that the total DNA dose is 20 µg. For example in mice receiving the 3 µg dose of the HBV replicon, 17 µg of the chosen eiRNA vector(s) is injected for a total of 20 µg injected DNA. The amount of this dose is therefore dependent upon the dose of HBV plasmid used. Control animals are injected with the HBV replicon but not with an eiRNA vector. Control mice are instead co-injected with an inert filler DNA, pGL3-basic (Promega, Madison, WI) such that the total amount of DNA in the formulation is 20 µg. eiRNA vectors in this study are the U6-based expression plasmids, e.g., Ambion, Inc., Austin, TX, USA. These vectors encode short hairpin RNAs derived from SEQ ID NO:1 and SEQ ID NO:4. The exact sequences encoded by these vectors are described below. The vectors are co-injected in equal amounts (by weight). It is expected that

similar results will be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

Description of U6-based eiRNA vector encoding sequences derived from SEQ ID NO:1: vector encodes a hairpin containing sequences mapping to coordinates 2905-2929 of accession #s V01460 and J02203 (i.e. the hairpin contains the sense and antisense version of this sequence, separated by a loop structure of TTCAAAAGA). Description of U6-based vector sequences can be found in Lee et al. [9]. The second eiRNA vector used in this experiment encodes a hairpin derived from SEQ ID NO:4 and encodes sequences mapping to coordinates 1215-1239 of Accession # V01460 and J02203.

Liver samples are taken from injected animals on day 1 following injection and analyzed for the presence of HBV RNA. This time point has been selected based on published results from Dr. Chisari's laboratory which detail the kinetics of HBVayw plasmid replication in mice following hydrodynamic delivery and demonstrates that peak RNA expression occurs in the liver on day 1 following hydrodynamic delivery [1]. The presence of HBV RNA in liver samples is ascertained by Northern blot analysis. Liver tissue will be evaluated for the down-regulation of HBV RNA expression. In addition, serum will be collected from day 4 mice for measurement of HBVsAg and DNA-containing viral particles. Assays will be as described for the cell culture replicon experiment (Example 3) and as in Yang et al. [1]. Each vector and control group will be comprised of 2 sets of animals, each set corresponding to a collection time point. There are 5 animals is each set.

Results:

Mice that are injected with the HBV replicon and the eiRNA constructs will have decreased HBV-specific RNA, and HBsAg and HBV viral particles as compared to the control animals. In individual animals, decreases will range from about 70% to near 100%.

EXPERIMENT 2

Transgenic mouse studies: Background.

We will be using the HBV transgenic mouse model developed in Dr. Chisari's laboratory [8]. These mice replicate appreciable amounts of HBV DNA and have demonstrated their utility as an antiviral screen that is a predictor of human efficacy [4]. These animals are also ideal in that they are a model for HBV-integrant-mediated expression of antigen and thus can serve as a model not only for viral replication but also for RT-independent expression of antigen. This is important as we are interested in targeting not only viral replication but integrant-mediated antigen expression as well.

These experiments differ from the hydrodynamic delivery experiments in that the effector plasmids are administered to animals using clinically relevant nucleic acid delivery methods. Effectiveness in this model demonstrates efficient delivery of the effector plasmids to mouse hepatocytes.

Experiment.

Mice described in reference [8] will be injected IV with a formulation containing the eiRNA vectors described in the hydrodynamic delivery example. These are the U6-based eiRNA vectors encoding hairpins containing sequences derived from SEQ ID NO:1 and SEQ ID NO:4.

Formulation of DNA to be injected.

DNA is formulated with trilactosyl spermine and cholesteryl spermine as described in PCT/US03/14288, "Methods for Delivery of Nucleic Acids", Satishchandran, filed 06-May-2003. Briefly, three formulations are made, all using a charge ratio of 1.2 (positive to negative charge). However, it should noted that formulations with charge ratios between 0.8 and 1.2 are all expected to exhibit efficacy. The DNA starting stock solution for each plasmid is 4 mg/ml. The two plasmid stock

solutions are mixed together in equal amounts such that each plasmid is at 2 mg/ml. This plasmid mixture is used for the final formulating. Formulation is as described in PCT/US03/14288 (above): Formulation A) 35% trilactosyl spermine, 65% cholesteryl spermine, Formulation B) 50% trilactosyl spermine, 50% cholesteryl spermine and Formulation C) 80% trilactosysl spermine, 20% cholesteryl spermine. All resultant formulations now contain each plasmid at 1 mg/ml.

Mice are IV injected with 100 µl formulated DNA. One group of mice receives Formulation A, a second group receives Formulation B and a third group receives Formulation C. Three groups of control mice are similarly injected with formulations containing a control DNA, pGL3Basic (Promega, Madison WI), Formulations D, E and F. Injections are carried out once a day for four consecutive days. Injecting for only 1-3 days is efficacious, however, more robust efficacy is seen with a four day injection protocol.

Following administration, HBV RNA and serum levels of HBsAg and DNA containing viral particles will be quantitated on days 5 and 9 post first injection. All analyses will be as described for the hydrodynamic delivery studies.

Results:

HBV-specific RNA levels, HBsAg and virus containing DNA particles will have decreased relative to controls in the Formulation A, B and C groups.

EXAMPLE #4

Silencing HBV replication and expression in a replication competent cell culture model

Brief description of cell culture model:

A human liver derived cell line such as the Huh7 cell line is transfected with an infectious molecular clone of HBV consisting of a terminally redundant viral genome that is capable of transcribing all of the

viral RNAs and producing infectious virus [1-3]. The replicon used in these studies is derived from the virus sequence found in Gen Bank Accession AF090840. Following internalization into hepatocytes and nuclear localization, transcription of the infectious HBV plasmid from several viral promoters has been shown to initiate a cascade of events that mirror HBV replication. These events include translation of transcribed viral mRNAs, packaging of transcribed pregenomic RNA into core particles, reverse transcription of pregenomic RNA, and assembly and secretion of virions and HBsAg particles into the media of transfected cells. This transfection model reproduces most aspects of HBV replication within infected liver cells and is therefore a good cell culture model with which to look at silencing of HBV expression and replication.

In this model, cells were co-transfected with the infectious molecular clone of HBV and an eiRNA construct. The cells were then monitored for loss of HBV expression and replication as described below.

The following is an example of an experiment that was performed using an eiRNA vector encoding sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. The particular eiRNA vector used for this experiment is T7 RNA polymerase-based and encodes a duplex RNA of about 650 bp (See e.g., WO 00/63364, filed April 19, 2000). It is expected that similar results would be obtained using other expression and promoter systems as described elsewhere herein and/or vectors encoding alternative structures (i.e. duplex).

Experimental Procedure: Transfection.

Huh7 cells were seeded into six-well plates such that they were between 80-90% confluency at the time of transfection. All transfections were performed using Lipofectamine™ (InVitrogen) according to the manufacturer's directions. In this experiment, cells were transfected with A) 50 ng of the infectious HBV plasmid adw subtype, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid in Example 3), and 1.5 µg of the HBV-specific eiRNA vector (described below); B) 50 ng of the

infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.5 µg of an irrelevant dsRNA expression vector; C) 125 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of the HBV-specific eiRNA vector; and D) 125 ng of the infectious HBV plasmid, 1 µg of the T7 RNA polymerase expression plasmid and 1.4 µg of an irrelevant dsRNA expression vector. All transfections were carried out in duplicate. In this experiment transfections B and D served as controls. Four days post-transfection, media was removed from transfected cells and assayed for the presence of HBsAg (see below). Media from untransfected cells was also assayed as a background control.

Monitoring cells for loss of HBV expression.

Following transfection, cells were monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion. Cells were monitored by assaying the media of transfected cells (and a media control) at 4 days post-dsRNA administration. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), was used to detect hepatitis B surface antigen (HBsAg). HBsAg was measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis ofHB sAg.

Results:

Cells transfected with the HBV-specific eiRNA construct exhibited an 82-93% decrease in HBsAg at the four-day timepoint relative to the control transfections.

HBV-specific eiRNA used in this experiment

The eiRNA vector encodes a dsRNA mapping to coordinates 2027-2674 of GenBank Accession # AF090840. The sequence therefore includes sequences derived from both SEQ ID NO:1 and SEQ ID NO:2. More specifically, the sequence includes all of SEQ ID NO:2 and 134 bp derived from SEQ ID NO:1.

EXAMPLE #5

The down-regulation of HCV in a cell culture replicon model

Brief description

In this experiment, a cell line is created which expresses functional HCV replicons. Creation of the cell line is as detailed in Lohmann et al. [10]. In this experiment Huh7 cells are used as the parental cell line but in theory any human hepatocyte derived cell line can be used. The cells are then transfected with an HCV specific eiRNA vector. The presence of HCV-specific RNA is ascertained by Northern blot analysis as described in Lohmann et al. [10] at days 3-7 post-transfection of eiRNA.

Experimental protocol: Transfection.

Huh7 cells expressing HCV replicons are seeded into six-well plates such that they are between 80-90% confluency at the time of transfection. All transfections are performed using Lipofectamine™ (InVitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 1 µg of a T7 RNA polymerase expression plasmid (plasmid described in Example 3) and 1.5 µg of a T7-based eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (vector described at end of example). Control cells are transfected with 1 µg T7 RNA polymerase expression plasmid and 1.5 µg of the HBV-specific (SEQ ID NO:1 specific) T7-based eiRNA vector described in Example 3. Untransfected replicon-expressing HuH 7 cells are included as a second control. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20

transfections (10 per mix). At days 3, 4, 5, 6, and 7, two wells of cells/each transfection are lysed and RNA is extracted using standard techniques. Samples are analyzed simultaneously by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

Results

Cells transfected with the HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed.

HCV-specific eiRNA vector.

The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters and encoding other types of RNA structures will have similar effects.

EXAMPLE #6

Treatment of an HBV/HCV co-infection

Brief description

In this example, cells that are replicating both HBV and HCV replicons are transfected with an eiRNA vector that encodes both HBV and HCV-specific eiRNA.

Experimental protocol:

Creation of cell lines that contain both HBV and HCV replicons.

HuH 7 cells are first engineered to express functional HCV replicons. Creation of the cell line is as detailed in Lohmann et al. [10]. After cell line establishment, the cells are transfected with an infectious

HBV replicon plasmid as described in Example 3 and below in the "Transfection of cells" section. In this example, the replicon is derived from the virus sequence found in Gen Bank Accession #s V01460 and J02203. Theoretically, it is also possible to first create a cell line that stably expresses the HBV replicon and then use this cell line to create one that also expresses HCV replicons. It is also possible to transfect the cells simultaneously with both the HBV and HCV replicons and select and expand cells that are replicating both HBV and HCV replicons.

Transfection of cells.

In this example, the HBV and HCV eiRNAs are encoded by separate cistrons within the same vector. However, similar results are expected if the eiRNAs are encoded within the same cistron or provided by separate vectors. In this example, transcription from each cistron is driven by the T7 RNA polymerase promoter and T7 RNA polymerase. Each promoter is followed by a hairpin eiRNA which in turn is followed by a T7 terminator (**FIG. 1**). The cistrons in this example are converging but one could also use diverging cistrons. It should also be noted that one could use other expression systems (including viral) to produce these RNAs and one could also use other promoters, e.g., as described elsewhere herein, to drive expression of these RNAs without significantly affecting efficacy. Selection of the appropriate expression systems and promoters is within the skill in this art. Also one could express other eiRNA structures, e.g., as described elsewhere herein, as well as others, described in the literature in this area. In this example, the HBV eiRNA vector encodes sequences derived from SEQ ID NO:1 and the HCV eiRNA vector encodes sequences derived from SEQ ID NO:11. Description of vector inserts is located at the end of this example.

Huh7 cells are seeded into six-well plates such that they are between 80-90% confluency at the time of transfection. All transfections are performed using Lipofectamine™ (Invitrogen) according to the manufacturer's directions. In this experiment, cells are transfected with 50

ng of the infectious HBV plasmid, 1 µg of a T7 RNA polymerase expression plasmid (description of plasmid is in Example 3), 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:1 (described below and in Example 3), and 600 ng of an eiRNA vector encoding a hairpin RNA comprised of sequences derived from SEQ ID NO:11 (described below). Control cells are transfected with 50 ng of the HBV plasmid and 1 µg of the T7 RNA polymerase expression plasmid. An inert filler DNA, pGL3-basic (Promega, Madison WI), is added to all transfections where needed to bring total DNA/transfection up to 2.5 µg DNA. Each transfection mix is made such that ten transfections can be performed/mix resulting in a total of 20 transfections (10 per mix).

Analyses.

Following transfection, cells are monitored for the loss or reduction in HBV expression and replication by measuring HBsAg secretion and DNA-containing viral particle secretion. Cells are monitored by assaying the media of transfected cells beginning at 2 days post dsRNA administration and every other day thereafter for a period of three weeks. The Auszyme ELISA, commercially available from Abbott Labs (Abbott Park, IL), is used to detect hepatitis B surface antigen (HBsAg). HBsAg is measured since it is associated not only with viral replication but also with RNA polymerase II initiated transcription of the surface Ag cistron in the transfected infectious HBV clone. Since HBsAg synthesis can continue in the absence of HBV replication it is important to down-regulate not only viral replication but also replication-independent synthesis of HBsAg. Secretion of virion particles containing encapsidated HBV genomic DNA is also measured. Loss of virion particles containing encapsidated DNA is indicative of a loss of HBV replication. Analysis of virion secretion involves a technique that discriminates between naked, immature core particles and enveloped infectious HBV virions [6]. Briefly, pelleted viral particles from the media of cultured cells are subjected to Proteinase K digestion to degrade the core proteins. Following inactivation of Proteinase K, the

sample is incubated with RQ1 DNase (Promega, Madison, WI) to degrade the DNA liberated from core particles. The sample is digested again with Proteinase K in the presence of SDS to inactivate the DNase as well as to disrupt and degrade the infectious enveloped virion particle. DNA is then purified by phenol/chloroform extraction and precipitated. HBV specific DNA is detected by gel electrophoresis followed by Southern Blot analysis.

At days 3, 4, 5, 6 and 7, two wells of cells/each transfection (experimental and control) are lysed and RNA is extracted using standard techniques. Samples are also analyzed by Northern blot analysis for the presence of HCV-specific RNA as described in Lohmann et al. [10].

Results.

Cells transfected with the HBV-HCV-specific eiRNA vector will show decreased HCV-specific RNA levels relative to the control cells at every time-point analyzed. In addition, the levels of HBsAg and HBV viral particles will also decrease relative to the control transfections.

HCV-specific eiRNA sequence.

The eiRNA vector is T7-based and encodes a hairpin RNA. One side of the hairpin comprises SEQ ID NO:48.

This sequence is followed by a loop structure of 9 Ts. The second side of the hairpin contains a sequence that is complementary to the first side of the hairpin. One skilled in the art can easily design and construct hairpin constructs. Note: it is anticipated that other types of eiRNA vectors driven by other promoters and encoding other types of RNA structures, including various hairpin structures will have similar effects. Especially desirable are, e.g., "forced" hairpin constructs, partial hairpins capable of being extended by RNA-dependent RNA polymerase to form dsRNA hairpins, as taught in USSN 60/399,998P, filed 31-Jul-2002 and PCT/US2003/024028, filed 31-Jul-2003, as well as the "udderly" structured hairpins (e.g., multi-hairpin long dsRNA vectors and multi-short hairpin structures), hairpins with mismatched regions, and multiepitope constructs

as taught in USSN 60/419,532, filed 18-Oct-2002, and PCT/US2003/033466, filed 20-Oct-2003, as well as a variety of other dsRNA structures known to those of skill in the art.

HBV-specific eiRNA-SEQ ID NO:1

The vector is T7-based as described above. The insert encodes a unimolecular hairpin comprised of sequences mapping from coordinate 3004-2950 (About 55 bp) of GenBank accession #s V01460 and J02203. One region of the hairpin encodes the sense version of the sequences and the second region of the hairpin encodes the antisense version of this sequence. Hairpins can easily be designed and made by those skilled in the art.

References

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CLAIMS

1. Use of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T, and wherein said double-stranded RNA is homologous in sequence to the target gene, for the manufacture of a medicament for inhibiting expression of a target polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell.

2. The use of claim 1, further comprising wherein effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:1 through SEQ ID NO:10 are suitable for administration to the same cell.

3. The use of claim 1, wherein one or more expression vectors comprising an expression construct capable of enabling production in said mammalian cell of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T, are suitable for administration.

4. The use of claim 3, wherein said one or more expression vectors further comprise at least one promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a U6 promoter, a tRNA promoter, and a mitochondrial promoter, said promoter operably linked to said at least 19 contiguous base pair nucleotide sequence.

5. Use of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T, for the manufacture of a medicament for inhibiting expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell.

6. The use of claim 5, further comprising wherein effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO: 27; wherein U is substituted for T; are suitable for administration to the same cell.

7. The use of claim 5, wherein one or more expression vectors comprising an expression construct capable of enabling production in said mammalian cell of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T, are suitable for administration.

8. The use of claim 6, wherein one or more expression vectors comprising an expression construct capable of enabling production in said mammalian cell of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; wherein U is substituted for T, are suitable for administration.

9. The use of claims 7 or 8, wherein said one or more expression vectors further comprise at least one promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a U6 promoter, a tRNA promoter, and a mitochondrial promoter, said promoter operably linked to said at least 19 contiguous base pair nucleotide sequence.

10. A use of a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T and wherein said double-stranded RNA is homologous in sequence to the target gene;

and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12; and SEQ ID NO:27; wherein U is substituted for T and wherein said double-stranded RNA is homologous in sequence to the target gene, for the manufacture of a medicament for inhibiting expression of both a target polynucleotide sequence of hepatitis B virus and a target polynucleotide sequence of hepatitis C virus in the same *in vivo* mammalian cell.

11. The use of claim 10, further comprising wherein effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than two of SEQ ID NO:1 through SEQ ID NO:12; and SEQ ID NO:27; are suitable for administration to the same cell.

12. The use of claim 10, wherein one or more expression vectors comprising an expression construct capable of enabling production in said mammalian cell of a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T; and a double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T, are suitable for administration.

13. The use of claim 12, wherein said one or more expression vectors further comprise: a first promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a U6 promoter, a tRNA promoter, and a mitochondrial promoter, said first promoter operably linked to said first at least 19 contiguous base pair nucleotide sequence; and a second promoter selected from an RNA polymerase I promoter, an RNA polymerase II promoter, a T7 polymerase promoter, an SP6 polymerase promoter, an RNA polymerase III promoter, a U6 promoter, a tRNA promoter, and a mitochondrial promoter, said second promoter operably linked to said second at least 19 contiguous base pair nucleotide sequence.

14. The use of any of preceding claims 1, 5 or 10 wherein the mammalian cell is a human cell.

15. A composition for inhibiting the expression of a target polynucleotide sequence of hepatitis B virus in an *in vivo* mammalian cell comprising at least one double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T and wherein said double-stranded RNA is homologous in sequence to the target gene.

16. The composition of claim 15, further comprising wherein double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:1 through SEQ ID NO:10 are present in the composition.

17. A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in an *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12; wherein U is substituted for T.

18. The composition of claim 17, further comprising wherein double-stranded RNA effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than one of SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:27; are present in the composition.

19. A composition for inhibiting the expression of both a target polynucleotide sequence of hepatitis B virus and a target polynucleotide sequence of hepatitis C virus in a single *in vivo* mammalian cell comprising a double-stranded RNA effector molecule comprising a first at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; wherein U is substituted for T and wherein said double-stranded RNA is homologous in sequence to the target gene; and a

double-stranded RNA effector molecule comprising a second at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:27; wherein U is substituted for T and wherein said double-stranded RNA is homologous in sequence to the target gene.

20. The composition of claim 19, further comprising wherein effector molecules comprising an at least 19 contiguous base pair nucleotide sequence from within more than two of SEQ ID NO: 1 through SEQ ID NO:12 and SEQ ID NO:27 are present in the composition.

21. The composition of any of claims 15, 17 or 19 wherein the mammalian cell is a human cell.

22. A composition for inhibiting the expression of a target polynucleotide sequence of hepatitis B virus in a mammalian cell, comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of:

a) SEQ ID NO:2;
b) SEQ ID NO:3;
c) SEQ ID NO:4;
d) SEQ ID NO:5;
e) SEQ ID NO:6;
f) SEQ ID NO:7;
g) SEQ ID NO:8;
h) SEQ ID NO:9;
i) SEQ ID NO:10;
j) the complement of (a) through (i); and
k) a mixture of (a) through (j).

23. A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of:

a) SEQ ID NO:11;
b) SEQ ID NO:12;
c) SEQ ID NO:27

d) the complement of (a), (b) or (c); and

e) a mixture of (a) through (d).

24. A composition for inhibiting the expression of a target polynucleotide sequence of hepatitis B virus and a target polynucleotide sequence of hepatitis C virus in the same mammalian cell, comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from the group consisting of:

a) SEQ ID NO:2;
b) SEQ ID NO:3;
c) SEQ ID NO:4;
d) SEQ ID NO:5;
e) SEQ ID NO:6;
f) SEQ ID NO:7;
g) SEQ ID NO:8;
h) SEQ ID NO:9;
i) SEQ ID NO:10;
j) SEQ ID NO:11;
k) SEQ ID NO:12
l) SEQ ID NO:27
m) the complement of a sequence of (a) through (l); and
n) a mixture of (a) through (m);

wherein said composition comprises at least one from the group (a) through (i) and one from the group (j), (k), and (l).

25. The composition of any of claims 22, 23 or 24 wherein said at least 19 contiguous nucleotide sequence comprises DNA and the mammalian cell is a human cell.

26. The composition of any of claims 22, 23 or 24 wherein said at least 19 contiguous nucleotide sequence comprises RNA and the mammalian cell is a human cell; and further wherein U is substituted for T.

27. A polynucleotide sequence comprising a sequence selected from SEQ ID NO:14 through SEQ ID NO:24.

28. A polynucleotide sequence comprising nucleotides 1-19, 1-20, 1-21, 2-20, 2-21, or 3-21 of a sequence selected from SEQ ID NO:14 through SEQ ID NO:24.

29. A polynucleotide sequence comprising an at least 19 contiguous base pair nucleotide sequence from within a sequence selected from SEQ ID NO:27 through SEQ ID NO:44.

30. A composition for inhibiting the expression of a polynucleotide sequence of hepatitis C virus in a mammalian cell, comprising a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from within SEQ ID NO:27; wherein U is substituted for T.

31. An expression construct capable of expressing a composition of any of claims 15-26 or 30 or a polynucleotide sequence of claims 27-29.

32. A mammalian cell comprising an expression construct of claim 31.

SEQUENCE LISTING

<110> Nucleonics, Inc.
Pachuk, Catherine
Satishchandran, C.
Zurawski, Vincent
Mintz, Liat

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<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 1737-1757 in Genebank accession # V01460

<400> 18
ggattcagcg ccgacgggac gagagaactt cgtcccgtcg gcgctgaatc c 51

<210> 19
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 1907-1927 in Genebank accession # V01460

<400> 19
ttccgcagta tggatcggca gagagaactt ctgccgatcc atactgcgga a 51

<210> 20
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 1912-1932 in Genebank accession # V01460

<400> 20
cagtatggat cggcagagga gagagaactt ctcctctgcc gatccatact g 51

<210> 21
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 1943-1963 in Genebank accession # V01460

<400> 21
tccacgcatg cgctgatggc cagagaactt ggccatcagc gcatgcgtgg a 51

<210> 22
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 1991-2011 in Genebank accession # V01460

<400> 22
tgcgtcagca aacacttggc aagagaactt tgccaagtgt ttgctgacgc a 51

<210> 23
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 2791-2811 in Genebank accession # V01460

<400> 23
aaaacgccgc agacacatcc aagagaactt tggatgtgtc tgcggcgttt t 51

<210> 24
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 2791-2811mut in Genebank accession # V01460

<400> 24
aaaacaccac acacgcatcc aagagaactt tggatgcgtg tgtggtgttt t 51

<210> 25
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 2912-2932 in Genebank accession # V01460

<400> 25
ttgagagaag tccaccacga gagagaactt ctcgtggtgg acttctctca a 51

<210> 26
<211> 51
<212> DNA
<213> Artificial

<220>
<223> eiRNA encoding sequence mapping to HBV-AYW coordinates 2919-2939 in Genebank accession # V01460

<400> 26

aagtccacca cgagtctaga cagagaactt gtctagactc gtggtggact t 51

<210> 27
<211> 101
<212> DNA
<213> Hepatitis C Virus

<400> 27
tttggtggct ccatcttagc cctagtcacg gctagctgtg aaaggtccgt gagccgcttg 60

actgcagaga gtgctgatac tggcctctct gcagatcaag t 101

<210> 28
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 28
gctaaacact ccaggccaat acctgtctc 29

<210> 29
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 29
tcctttggtg gctccatctt acctgtctc 29

<210> 30
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 30
gctccatctt agccctagtc acctgtctc 29

<210> 31
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 31
tcttagccct agtcacggct acctgtctc 29

<210> 32

<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 32
cctagtcacg gctagctgtg acctgtctc 29

<210> 33
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 33
ctagtcacgg ctagctgtga acctgtctc 29

<210> 34
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 34
cgtgagccgc ttgactgcag acctgtctc 29

<210> 35
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 35
gctgatactg gcctctctgc acctgtctc 29

<210> 36
<211> 29
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 36
actggcctct ctgcagatca acctgtctc 29

<210> 37
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 37
ctggcctctc tgcagatcaa g 21

<210> 38
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 38
tgcagagagt gctgatactg g 21

<210> 39
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 39
tgagccgctt gactgcagag a 21

<210> 40
<211> 20
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 40
gaaaggtccg tgagccgctt 20

<210> 41
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 41
tagctgtgaa aggtccgtga g 21

<210> 42
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 42
ttagccctag tcacggctag c 21

<210> 43
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 43
tccatcttag ccctagtcac g 21

<210> 44
<211> 21
<212> DNA
<213> Artificial

<220>
<223> siRNA encoding sequence mapping to X region of Hepatitis C Virus

<400> 44
ttggtggctc catcttagcc c 21

<210> 45
<211> 21
<212> RNA
<213> Hepatitis C Virus

<400> 45
aaccucaaag aaaaaccaaa c 21

<210> 46
<211> 21
<212> RNA
<213> Artificial

<220>
<223> lamin siRNA

<400> 46
aacuggacuu ccagaagaac a 21

<210> 47
<211> 2652
<212> DNA
<213> Bacteriophage T7

<400> 47
atgaacacga ttaacatcgc taagaacgac ttctctgaca tcgaactggc tgctatcccg 60

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catgagtctt acgagatggg tgaagcacgc ttccgcaaga tgtttgagcg tcaacttaaa 180

gctggtgagg ttgcggataa cgctgccgcc aagcctctca tcactaccct actccctaag 240

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gtactggctg atttctacga ccagttcgct gaccagttgc acgagtctca attggacaaa 2580

atgccagcac ttccggctaa aggtaacttg aacctccgtg acatcttaga gtcggacttc 2640

gcgttcgcgt aa 2652

<210> 48
<211> 323
<212> DNA
<213> Artificial

<220>
<223> T7 polymerase-based eiRNA

<400> 48
atcactcccc tgtgaggaac tactgtcttc acgcagaaag cgtctagcca tggcgttagt 60

atgagtgtcg tgcagcctcc aggacccccc ctcccgggag agccatagtg gtctgcggaa 120

ccggtgagta caccggaatt gccaggacga ccgggtcctt tcttggatga acccgctcaa 180

tgcctggaga tttgggcgtg cccccgcgag actgctagcc gagtagtgtt gggtcgcgaa 240

aggccttgtg gtactgcctg atagggtgct tgcgagtgcc ccgggaggtc tcgtagaccg 300

tgcaccatga gcacaaatcc taa 323