

Inhibition of hepatitis B virus in mice by RNA interference

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Hepatitis B virus (HBV) infection substantially increases the risk of chronic liver disease and hepatocellular carcinoma in humans. RNA interference (RNAi) of virus-specific genes has emerged as a potential antiviral mechanism. Here we show that RNAi can be applied to inhibit production of HBV replicative intermediates in cell culture and in immunocompetent and immunodeficient mice transfected with an HBV plasmid. Cotransfection with plasmids expressing short hairpin RNAs (shRNAs) homologous to HBV mRNAs induced an RNAi response. Northern and Southern analyses of mouse liver RNA and DNA showed substantially reduced levels of HBV RNAs and replicated HBV genomes upon RNAi treatment. Secreted HBV surface antigen (HBsAg) was reduced by 94.2% in cell culture and 84.5% in mouse serum, whereas immunohistochemical detection of HBV core antigen (HBcAg) revealed >99% reduction in stained hepatocytes upon RNAi treatment. Thus, RNAi effectively inhibited replication initiation in cultured cells and mammalian liver, showing that such an approach could be useful in the treatment of viral diseases.

RNA interference is a process during which cytoplasmic long double-stranded RNAs (dsRNAs) produced by viral infection, by transposons or by introduced transgenes are targeted for inactivation¹. These long dsRNAs are processed into 21- to 23-nucleotide (nt) guide RNA duplexes by an RNase called Dicer² and are further incorporated into an RNA-induced silencing complex (RISC)³. The RISC complex uses the guide RNAs to identify homologous RNAs in the cell and proceeds to cleave them. Synthetic small interfering RNAs (siRNAs)⁴ and shRNAs transcribed *in vivo* from DNA templates trigger specific silencing of genes when transfected into cultured cells⁵. Several groups have inhibited replication of viruses in culture using RNAi^{6–12}, including inhibition of HBV in cultured cells¹³. However, there are no reports of *in vivo* viral inhibition by RNAi in mammals. Thus, it remains unclear how RNAi might function in the context of the functional innate and adaptive immune systems present in whole animals. Here we examine the antiviral effect of shRNAs targeting HBV in mice.

Recently, we showed that synthetic siRNAs as well as shRNAs transcribed *in vivo* from DNA templates were potent inhibitors of gene expression in adult mice. Furthermore, we showed that a sequence from the hepatitis C virus (HCV), fused to a reporter gene, could be targeted by RNAi, suggesting the possibility of using RNAi as a therapeutic tool¹⁴. However, only a portion of the viral genome was present, and viral replication did not occur in that particular HCV model system. In contrast, many of the steps in the viral replication cycle of HBV occur in mice after transfection with plasmids containing the HBV genome¹⁵. This model system offers the opportunity to test the ability of RNAi to inhibit replication of a virus in a small animal model of human disease. Here we demonstrate that expressed shRNAs targeting

HBV mRNAs inhibited the steps in HBV replication that occur in cultured cells and in immunocompetent and immunodeficient mice.

RESULTS

Selection of RNAi target sites

Seven RNAi target sequences were chosen on the basis of their conservation among the major HBV genotypes¹⁶. In some cases, these sequences targeted overlapping reading frames of the virus such that multiple viral RNAs would be inhibited by one shRNA (Fig. 1a). Each shRNA targets the pregenomic RNA serving as the template for HBV genomic replication as well as the mRNA for the core antigen and the polymerase. shRNAs HBVU6no.1, HBVU6no.2, HBVU6no.3 and HBVU6no.4 also target the HBV S-antigen mRNAs. HBVU6no.5 also targets the X region and its transcript, whereas HBVU6no.6 and HBVU6no.7 target the pregenomic RNA in the overlap region encoding the core antigen and the polymerase. A schematic of the U6 shRNA expression cassette (Fig. 1b) and, as an example, the predicted folding of HBVU6no.2 (Fig. 1c) are shown.

HbsAg expression in cell culture is inhibited by HBV RNAi

To test whether RNAi could inhibit HBV in cell culture, a cotransfection assay was conducted. In each experiment three plasmids were cotransfected into cultured HuH-7 cells (a human hepatoma cell line): (i) 4 µg of the plasmid pTHBV2 (ref. 17) containing the HBV genome with some sequences duplicated to allow complete expression of all genes; (ii) 5 µg of a U6 shRNA expression vector, or either empty vector that does not express shRNAs or an shRNA vector targeting HCV (negative control RNAi) as negative controls; and (iii) 5 µg of a plasmid

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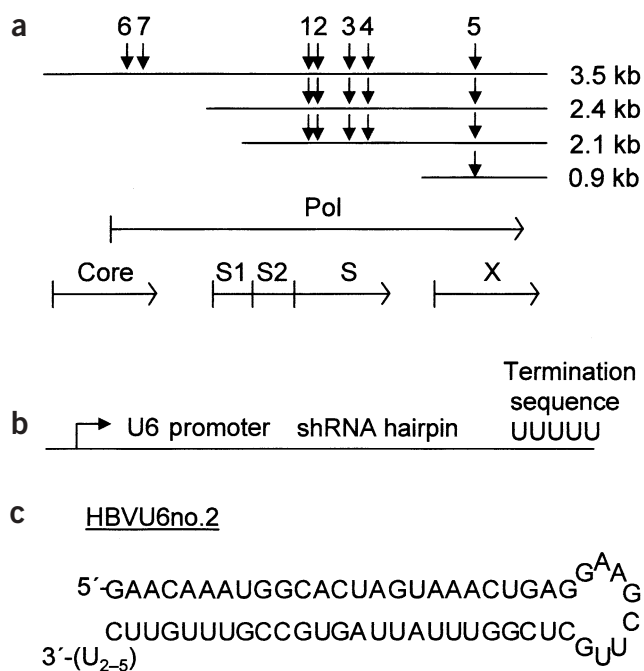


Figure 1 Target site and vector information. (a) Location of RNAi target sites. Downward arrows indicate the location of RNAi target sites within the four HBV transcripts. The 3.5-kb transcript is the pregenomic RNA that serves as the template for HBV viral DNA replication. The HBV open reading frames are shown below aligned with the HBV mRNAs. Pol, polymerase; Core, HBCAg; S1, large pre-surface antigen; S2, middle pre-surface antigen; S, HBsAg; X, X gene. The numbers above the arrows indicate the shRNA that targets that site. (b) Schematic of U6 promoter constructs. Transcription ends at a string of 5 Us. (c) Predicted folding of HBVU6no.2.

(pThAAT) that expresses the secreted protein human α 1-antitrypsin (hAAT)¹⁸. Transfection with pTHBV2 initiated an HBV replication cycle, resulting in production of replicated HBV genomes as well as viral mRNAs and proteins (including HBsAg and HbcAg). ELISA measurements of secreted hAAT¹⁹ monitored for transfection efficiency and nonspecific translational inhibition or toxicity. Average hAAT concentrations in culture medium at days 3 and 7 were similar in all groups.

On days 3, 6 and 8, HBsAg levels in the medium were measured (Fig. 2). With the exception of HBVU6no.1 (data not shown), treatment with each of the shRNA expression plasmids reduced the amount of HBsAg compared with the untreated control group, in three independent experiments. Treatment with HBVU6no.2 and HBVU6no.6 gave the greatest reduction in HBsAg ($94.2 \pm 0.59\%$ ($P = 0.0003$) and $91.5 \pm 1.4\%$ ($P = 0.0003$) on day 8, respectively), compared with the empty-vector control. These results demonstrate that RNAi can substantially inhibit HBV viral protein expression and therefore could ultimately suppress viral replication in cultured cells. The best inhibitors (HBVU6no.2 and HBVU6no.6) were studied further in mice.

HBV RNAs in mice are reduced by HBV shRNAs

Northern hybridization analysis was conducted to determine if treatment with HBVU6no.2 and HBVU6no.6 resulted in a reduction in the amount of HBV RNAs present in immunocompetent (C57BL/6J, Fig. 3a) or immunocompromised NOD/LtSz-Prkdc^{scid}/J (NOD SCID, Fig. 3b) mice. DNAs were transfected into mouse liver by hydrodynamic transfection^{20,21}, a method that results in gene transfer into

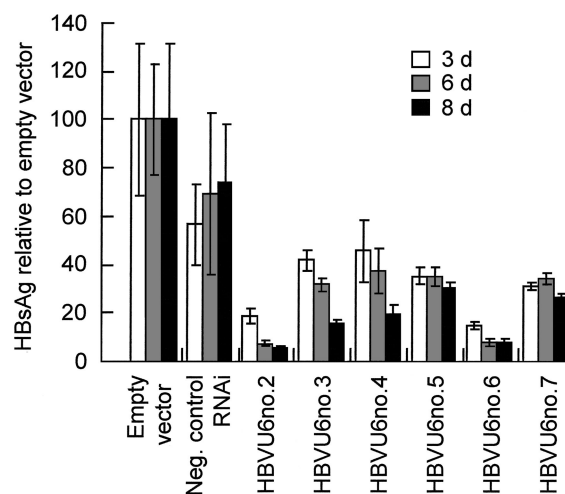


Figure 2 HBsAg measurements in medium of shRNA-treated cultured cells. HBsAg levels in culture medium are significantly reduced by treatment with HBV RNAi. Standard errors are shown. Observed reductions in HBsAg did not result from poor transfection, nonspecific inhibition of translation or toxicity, because the average hAAT levels for experimental groups were not reduced compared to the control group.

5–40% of mouse hepatocytes¹⁴. Mice were transfected with 12 μ g of pTHBV2, 5 μ g of pThAAT and 5 μ g of a control plasmid or shRNA expression plasmid. At day 7, total liver RNA was analyzed by northern hybridization. No bands were detected in total RNA from a naive mouse that was not transfected with pTHBV2 (Fig. 3a, lane 1). In total RNA samples from mice that received pTHBV2 (Fig. 3a, lanes 2–9; Fig. 3b, lanes 1–8), a 3.5-kilobase (kb) pregenomic RNA encoding the viral core and polymerase proteins was observed. RNAs of 2.4 kb and 2.1 kb encoding the viral envelope proteins were also observed; they comigrated in most lanes, although in a sample from an HBV transgenic mouse¹⁷, these two bands were barely resolved (Fig. 3a, lane 16). Consistent with a previous report¹⁵, the transcript encoding the X protein was not observed. Northern blotted membranes were also probed for transcript of the endogenous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as a loading control.

Substantial amounts of HBV RNAs were observed in total RNA from mice that did not receive an RNAi plasmid (No RNAi group, Fig. 3a, lanes 2 and 3; Fig. 3b, lanes 1 and 2). Mice that received a negative control shRNA plasmid (Neg. control RNAi) targeting HCV also showed substantial amounts of HBV RNAs (Fig. 3a, lanes 4 and 5; Fig. 3b, lanes 3 and 4). Compared with mice from the No RNAi group, immunocompetent and immunocompromised mice treated with HBVU6no.2 had 77% and 92% less HBV RNA, respectively, after normalization to the GAPDH endogenous transcript (Fig. 3a, lanes 6 and 7, Fig. 3b, lanes 5 and 6). Compared with the control No RNAi group, HBVU6no.6-treated immunocompetent (Fig. 3a, lanes 8 and 9) and immunocompromised (Fig. 3a, lanes 7 and 8) mice had reductions in HBV RNA of 31% and 58%, respectively. Thus, treatment with HBV shRNAs reduces HBV RNA transcripts.

HBVU6no.2 targets all three viral RNAs; however, HBVU6no.6 targets only the 3.5-kb transcript and should not reduce the levels of the 2.1- and 2.4-kb RNAs. HBVU6no.6 reduces all viral transcripts at the dose used, suggesting that some mechanism other than RNAi must be operating. This effect is observed in immunocompromised mice, suggesting that it does not result from an antigen-dependent immune response. The implications of these results are discussed later.

Replicated HBV genomes were reduced by HBV RNAi

Hydrodynamic transfection with pTHBV2 results in the production of HBV DNA-replicative intermediates in mice. Single-stranded (ss) and double-stranded (ds) replicative intermediates were observed (Fig. 4a, lane 17) for a replicative intermediate marker derived from a cell line that stably expresses HBV DNAs. However, no closed circular DNA (cccDNA) was observed in a similar hydrodynamic HBV model¹⁵, suggesting that the full viral replication cycle cannot be completed in mice. Analysis of our DNA samples also failed to detect cccDNA (Supplementary Fig. 1 online). Nonetheless, this model recapitulates most of the steps of HBV viral replication and thus serves as a good means for the evaluation of inhibitors of HBV replication. NOD SCID mice received 12 µg of pTHBV2 as well as 5 µg of one of the following: (i) no RNAi plasmid, (ii) an HCV shRNA expression plasmid (Neg., control RNAi), (iii) pHBVU6no.2 or (iv) pHBVU6no.6. Mice were killed at day 7 and total DNA extracted.

To determine definitively if treatment with HBVU6no.2 and HBVU6no.6 resulted in a reduction in replicated viral genome intermediates in the livers of mice, a modified Southern hybridization assay was conducted that detects replicated viral genomes (*DpnI* insensitive) but not bacterially methylated input plasmid (*DpnI* sensitive). Some DNA samples were not treated with *DpnI* (Fig. 4a), allowing visualization of the input plasmid. The same DNA samples were run on a separate gel and the copy number per cell of the input plasmid, pTHBV2, was determined by Southern hybridization using a probe for the ampicillin resistance gene present on this plasmid (copy number listed in Fig. 4a). The same DNA samples were then treated with *DpnI* to degrade input plasmid (Fig. 4b). Conditions that resulted in complete digestion of at least five copies per cell of input plasmid were used, which is sufficient to degrade all the bacterially methylated input DNA (Fig. 4b, lane 17). The amount of replicated DNA was quantified by phosphorimager analysis.

Replicated HBV genomic DNA was present in samples from mice in the control groups that did not receive an RNAi plasmid (Fig. 4a,b, lanes 5–7). Mice that received the negative control RNAi (Fig. 4a,b, lanes 8–10) had slightly less replicated DNA than the No RNAi group. DNA from HBVU6no.2-treated mice (Fig. 4a,b, lanes 11–13) had undetectable levels of replicated HBV genomes. HBVU6no.6-treated mice (Fig. 4a,b, lanes 14–16) had somewhat fewer replicated HBV DNA molecules. The Southern blotted membrane was stripped and reprobed for the endogenous mouse (*agouti*) gene, as a means of verifying that the observed difference in replicated HBV genomes did not result from variation of integrity of DNA samples loaded on the gels. Variation in the copy number of the HBV plasmid also did not account for the observed differences in replicated HBV genomes.

In contrast with the No RNAi group, ssHBV genome was the predominant molecular form in RNAi-treated mice (including the negative

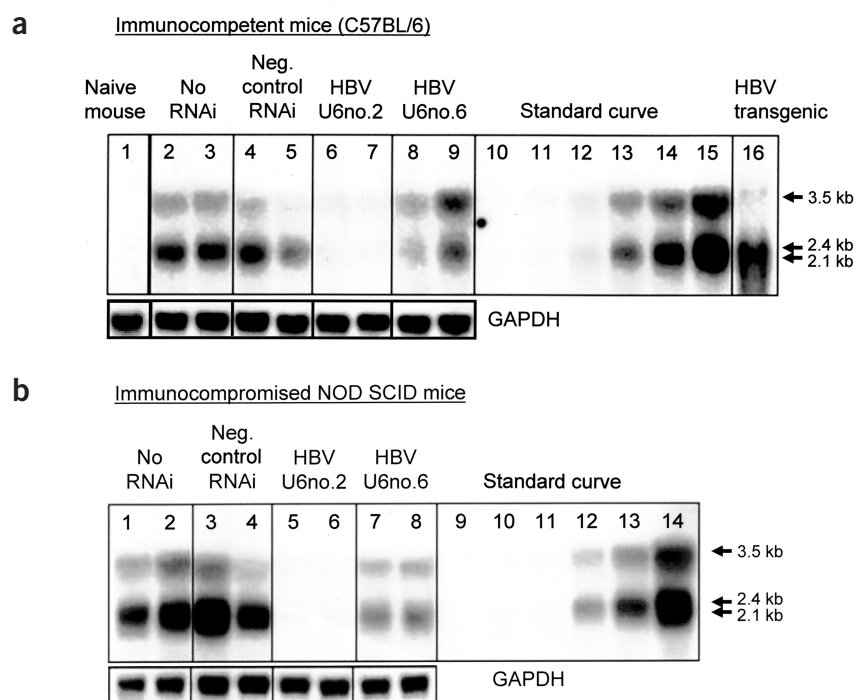


Figure 3 Northern hybridization analysis of HBV RNAs. **(a)** Samples from immunocompetent mice (C57BL/6). **(b)** Samples from immunocompromised NOD/LtSz-*Prkdc^{scid}/J* (NOD SCID) mice. Lane 1 in **a**, no bands are detected in RNA from a naive mouse (not transfected with pTHBV2). Lanes 2–9 in **a**, HBV pregenomic and HBsAg mRNAs are observed. Reported HBV RNA levels were normalized with the levels of the endogenous GAPDH transcript. Compared to mice from the No RNAi group (lanes 2 and 3 in **a**, lanes 1 and 2 in **b**), mice from the Neg. control RNAi group (lanes 4 and 5 in **a**, lanes 3 and 4 in **b**) had similar normalized amounts of HBV RNA. Mice that received HBVU6no.2 (lanes 6 and 7 in **a**, lanes 5 and 6 in **b**) had substantially reduced amounts of HBV RNA. Mice that received HBVU6no.7 also had reduced levels of both the 3.5-kb pregenomic RNA and the HBsAg mRNAs (lanes 8 and 9 in **a**, lanes 7 and 8 in **b**). Lane 16 in **a** shows total RNA from an HBV transgenic mouse. The HBV 3.5-kb pregenomic RNA as well as the 2.4- and 2.1-kb HBsAg mRNAs were observed. An endogenous GAPDH transcript controls for loading integrity of the RNA.

control RNAi). We cannot exclude the possibility that low levels of nuclease activity converted partially dsDNA to ssDNA. Nevertheless, RNAi directed against HBV results in substantial reduction in all of the steps of replication of HBV genomes that occur in immunocompromised mice. Because these experiments were conducted in NOD SCID mice, the observed reductions in HBV genomes did not result from cytotoxic T cell-mediated events. We carried out the same analysis using *DpnI*-treated DNA from immunocompetent BALB/c mice and also observed substantial reductions in HBV genomes (data not shown). Thus in both immunodeficient and immunocompetent mice, treatment with HBV shRNAs resulted in substantial reduction in all of the HBV-replicative forms produced in mice.

HBsAg expression in mice is inhibited by HBV RNAi

The same three-plasmid cotransfection model was used to test RNAi inhibition of HBsAg production in mice. Yang *et al.* reported that upon hydrodynamic transfection with an HBV plasmid in immunocompetent mice, HBsAg-neutralizing antibodies were observed starting at day 7 (ref. 15). To avoid complications associated with neutralizing antibodies, experiments were carried out in NOD SCID mice lacking B and T lymphocytes. Serum HBsAg was measured at day 4 and day 7 (Fig. 5).

At day 4, serum HBsAg for the Neg. control RNAi group and the HBV RNAi.6 group was similar to that of the No RNAi group

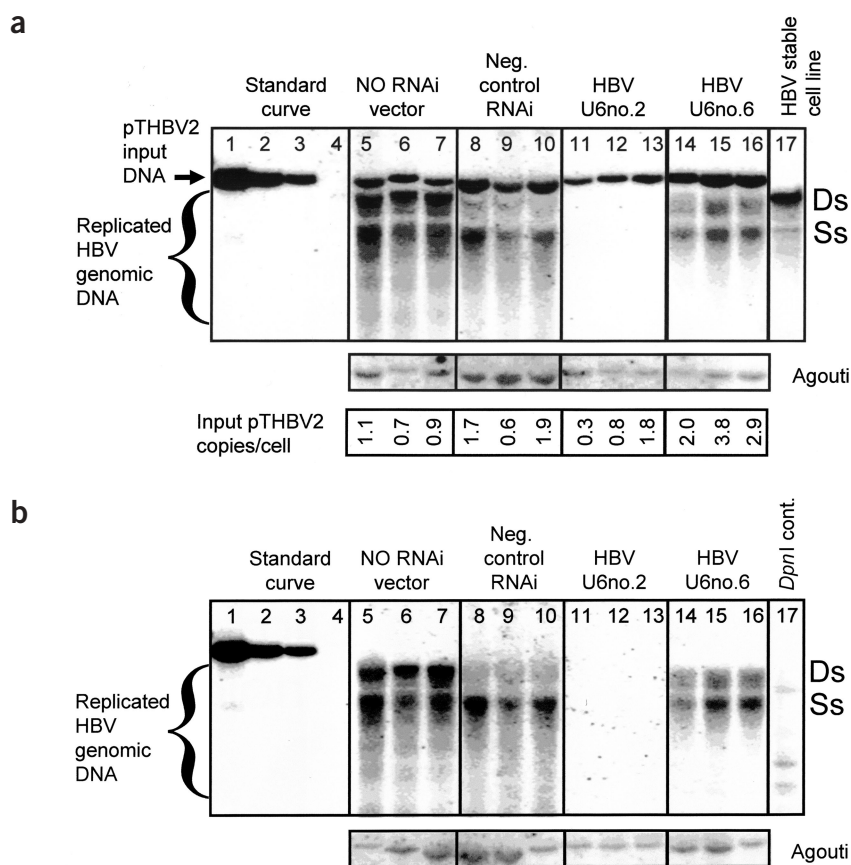


Figure 4 Southern hybridization analysis of HBV DNAs. **(a,b)** Southern hybridization analysis of HBV genomic DNA from mice that received (i) no RNAi plasmid, (ii) an HCV shRNA expression plasmid (Neg. control RNAi), (iii) pHBVU6no.2 or (iv) pHBVU6no.6. **(a)** Input ~7.3-kb plasmid is observed in total DNA samples that were treated only with *SacI* but not with *DpnI*. **(b)** To remove input plasmid DNA and leave only replicated HBV genomes, total DNA samples were treated with *DpnI* together with *SacI*. *DpnI* cleaves the bacterially methylated input DNA (pTHBV2) in 34 locations but does not cleave unmethylated replicated HBV genomes. Total DNA samples from mice that received 12 μ g of pTHBV2 and no RNAi plasmid (lanes 5–7 in **a** and **b**), an HCV shRNA Neg. control RNAi (lanes 8–10 in **a** and **b**), pHBVU6no.2 (lanes 11–13 in **a** and **b**) or pHBVU6no.6 (lanes 14–16 in **a** and **b**) are shown. Replicated HBV genomes were undetectable in DNA samples from mice that received HBVU6no.2 and were substantially reduced in mice that received pHBVU6no.6. A standard curve was generated by spiking 25, 5, 1 or 0 copies/cell of *SacI*-digested pTHBV2 into 10 μ g of total mouse DNA (lanes 1–4 in **a** and **b**, respectively). To ensure that the *DpnI* digestion conditions used to degrade the input DNA were sufficient, 5 copies/cell of pTHBV2 were spiked into 10 μ g of total mouse DNA (lane 17 in **b**) and digested in the same way. Replicated HBV ds and ssDNA is observed in DNase I-resistant cytoplasmic DNA extracted from 2.2.15 cells that stably express HBV genomic DNAs²⁴.

($P = 0.793$ and $P = 0.971$, respectively). In contrast, serum HBsAg of HBVU6no.2-treated mice at day 4 was reduced by 88% ($\pm 2.2\%$; $P = 0.0119$) compared with the No RNAi group. Compared to the No RNAi group, at day 7, serum HBsAg for the Neg. control RNAi group was statistically similar ($P = 0.194$), whereas HBsAg for the HBVU6no.2 and HBVU6no.6 groups was reduced by 85% ($\pm 3.3\%$; $P < 0.0001$) and 47% ($\pm 9.3\%$; $P = 0.0088$), respectively.

At days 4 and 7, serum hAAT was measured to ensure that transfection efficiencies were similar and to exclude the possibility of nonspecific translational arrest resulting from treatment with shRNAs. Average hAAT amounts varied by no more than twofold and declined by similar amounts for all groups between day 4 and day 7. These results demonstrated that HBVU6no.2 and pHBVU6no.6 shRNAs substantially reduce serum HBsAg in mice. Furthermore, the decrease in serum HBsAg was not a result of nonspecific translational arrest resulting from expression of short duplex RNAs, because hAAT levels were similar in all groups. The observation that HBVU6no.6 reduces serum HBsAg was unexpected, because it does not target the HBsAg mRNA; however, this observation correlates well with the observed reduction in HBsAg mRNA upon treatment with HBVU6no.6.

HBcAg expression in mice is inhibited by RNAi

HBcAg, the nucleocapsid protein, is synthesized in infected cells and is required for HBV viral replication. At day 7, paraffin-fixed liver sections were prepared from mice transfected with 5 μ g HBVU6no.2, HBVU6no.6 or empty vector as well as 4 μ g pTHBV2. Consistent with the expected transfection efficiency, $5.2 \pm 1.1\%$ of cells stained for HBcAg in tissue sections from mice that received the empty vector and pTHBV2 (Fig. 6a,b). Liver sections from mice receiving HBVU6no.2

had significantly reduced numbers of HBcAg-stained cells (reduced by $99.7 \pm 0.3\%$; $P = 0.0011$). Most fields contained no stained cells (Fig. 6c), although there were rare hepatocytes with lightly stained nuclei (Fig. 6d). The number of stained hepatocytes in sections from HBVU6no.6-treated mice was reduced by $94 \pm 1.9\%$ ($P = 0.0014$) (Fig. 6e). No staining was seen in sections from mice that did not receive pTHBV2 (Fig. 6f). These results demonstrate that HBV RNAi can inhibit the production of HBV proteins.

DISCUSSION

There has been considerable interest in the use of RNAi therapeutics to treat a wide range of diseases. Recent reports that RNAi can inhibit viral replication in cell culture support this notion^{6–13}. Previously, we demonstrated RNAi was functional in mice and could target sequences from HCV¹⁴. Recently, similar methods were used to inhibit the production of Fas receptor in mice. Fas siRNA treatment protected mice from fulminant hepatitis induced by injecting agonistic Fas-specific antibody²². These reports demonstrate the RNAi can be used therapeutically in mammals. However, to date there has been no report of RNAi inhibition of a virus in mammals. Here, we show that RNAi can inhibit all the steps of HBV replication that occur in cell culture and in mice. Thus, it may be possible to use shRNAs directed against the viral or host genes, to inhibit viral replication, and inhibit or reverse the fibrosis associated with the development of liver cirrhosis.

Four separate lines of evidence establish that RNAi substantially inhibited HBV in mice: (i) RNAi expression significantly reduced secreted HBsAg in culture medium and in mouse serum; (ii) HBV RNAs were substantially reduced in mouse liver; (iii) HBV genomic DNA was reduced to undetectable levels in mouse liver; and (iv) the

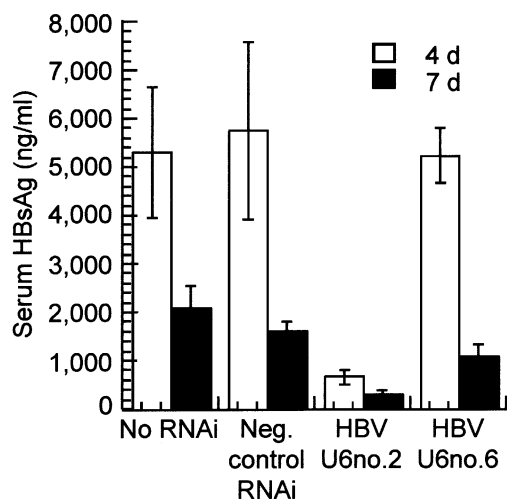


Figure 5 Serum HBsAg levels in shRNA-treated mice. HBsAg levels in NOD SCID mouse sera are significantly reduced after treatment with the shRNA expression plasmids HBVU6no.2 and HBVU6no.6 (error bars indicate standard errors).

number of cells staining for HBcAg was substantially decreased (intensity of staining was also decreased).

HBVU6no.6 reduced the levels of the 2.4- and 2.1-kb RNAs and serum HBsAg even though it does not directly target these RNAs. Southern analysis showed a difference in the ratio of HBV-replicative molecular forms upon treatment with shRNAs. These data raise the possibility that shRNAs may have some sequence-independent antiviral effects at the dose used. Further studies will be required to determine the mechanism by which this occurs. As with other drugs, it is likely that there is a therapeutic window in which RNAi is effective but does not cause undesirable side effects. Further studies will also be required to determine if, at the optimal doses of RNAi, non-sequence-specific effects can be minimized or eliminated.

RNAi can theoretically be directed to cleave any target RNA, providing a single methodology for rational drug design for many different diseases. For this reason RNAi has generated substantial interest. It is clear from our study that inhibition of viral replication by RNAi in mammals is feasible. In the present study, six out of seven RNAi inhibitors tested showed some antiviral effect, and two out of seven were very potent inhibitors. However, a recent study found poliovirus escape mutants after extended treatment with siRNAs¹¹. This suggests that multiple viral sequences must be targeted simultaneously so as to prevent the emergence of resistant strains.

METHODS

Plasmids. pTHBV2 (ref. 17) contains the HBV genome plus a redundancy for the sequences between nt 1067 and 1996 of the HBV genome. shRNAs were cloned downstream of the human U6 promoter as described²³. Additional information on cloning and general structure of shRNA vectors can be found at http://katahdin.cshl.org:9331/RNAi/docs/Web_version_of_PCR_strategy1.pdf.

Target sequences are as follows:

HBVU6no.1, 5'-TCGTGGTGGACTTCTCTCAATTTTC-3'
 HBVU6no.2, 5'-CTCAGTTTACTAGTGCCATTTGTTC-3'
 HBVU6no.3, 5'-ATGATGTGGTATTGGGGGCCAAGTC-3'
 HBVU6no.4, 5'-TGGCCAAAATTCGCAGTCCCAACC-3'
 HBVU6no.5, 5'-TCCCGTCTGTGCCTTCTCATCTGC-3'
 HBVU6no.6, 5'-CCTAGAAGAAGAATCCCTCGCCTC-3'
 HBVU6no.7, 5'-AGAAGATCTCAATCTCGGGAATCTC-3'

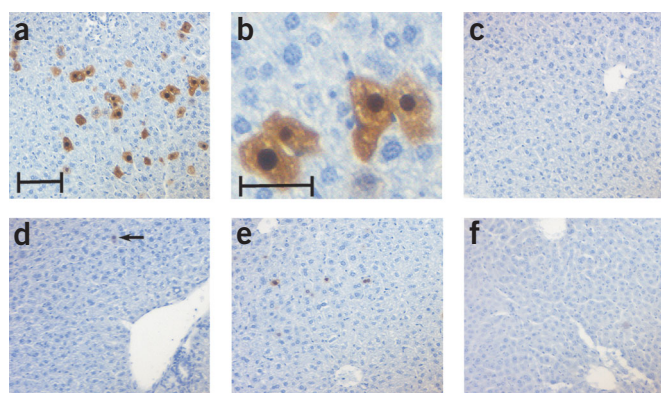


Figure 6 Immunohistochemical staining for HBcAg in liver sections. (a–f) Treatment with HBVU6no.2 or HBVU6no.6 reduces the number of cells that stain for HBcAg. Immunohistochemical staining for HBcAg was done on tissue sections from three animals per group. Representative sections are shown (four sections per animal were stained and counted). Paraffin-embedded sections from animals that received 4 μg pTHBV2, 3 μg pTHAAT and 5 μg of indicated plasmid. (a) Empty vector (10× magnification, scale bar 100 μm). (b) Empty vector (40× magnification, scale bar 10 μm). (c) HBVU6no.2; no stained cells were observed in most fields. (d) HBVU6no.2; faintly stained cell indicated by an arrow. (e) HBVU6no.6; some stained cells can be seen but staining is less intense than with empty vector. (f) Liver section from a mouse that did not receive any pTHBV2. No staining was observed.

Negative control RNAi, 5'-TGGATATGCACGGTGTGACTGATT-3' pThAAT and pEmpty vector are described in refs. 18 and 14, respectively.

Statistical methods. A one-way ANOVA analysis with a post-hoc Fisher's test was conducted.

Cell culture and mouse transfections. Calcium phosphate transfections were carried out using standard methods. Hydrodynamic transfections of plasmids in PBS were carried out as described^{20,21}. Mice that expressed very low levels of hAAT were considered poorly transfected and were excluded before analyses for HBV levels. Female BALB/c, C57BL/6J and NOD/LtSz-Prkdc^{scid}/J mice (18–22 g) were from Jackson Laboratory. Mice were treated according to NIH Guidelines for Animal Care and the Guidelines of Stanford University.

For descriptions of remaining procedures, see **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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