Hepatocyte-targeted RNAi Therapeutics for the Treatment of Chronic Hepatitis B Virus Infection

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RNA interference (RNAi)-based therapeutics have the potential to treat chronic hepatitis B virus (HBV) infection in a fundamentally different manner than current therapies. Using RNAi, it is possible to knock down expression of viral RNAs including the pregenomic RNA from which the replicative intermediates are derived, thus reducing viral load, and the viral proteins that result in disease and impact the immune system’s ability to eliminate the virus. We previously described the use of polymer-based Dynamic PolyConjugate (DPC) for the targeted delivery of siRNAs to hepatocytes. Here, we first show in proof-of-concept studies that simple coinjection of a hepatocyte-targeted, N-acetylgalactosamine-conjugated melittin-like peptide (NAG-MLP) with a liver-tropic cholesterol-conjugated siRNA (chol-siRNA) targeting coagulation factor VII (F7) results in efficient F7 knockdown in mice and nonhuman primates without changes in clinical chemistry or induction of cytokines. Using transient and transgenic mouse models of HBV infection, we show that a single coinjection of NAG-MLP with potent chol-siRNAs targeting conserved HBV sequences resulted in multilog repression of viral RNA, proteins, and viral DNA with long duration of effect. These results suggest that coinjection of NAG-MLP and chol-siHBVs holds great promise as a new therapeutic for patients chronically infected with HBV.

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INTRODUCTION

According to the World Health Organization, 360 million people globally are chronically infected with hepatitis B virus (HBV) and between 500,000 and 1 million a year die as a result of hepatocellular carcinoma, cirrhosis of the liver or liver failure caused by HBV. HBV is transmitted through the blood and infects hepatocytes in the liver. One-third of the global population becomes infected with HBV at some point in their lives. Those who are infected as adults are frequently able to mount a successful acute immune response that will control the infection. People who are infected with HBV as neonates or young children usually become chronically infected. In the immune tolerant phase of chronic infection, which can last for many years, the infected person typically produces very high levels of viral DNA and viral antigens. However, the infection is not cytotoxic and the carrier initially has no signs of disease. Over time, the chronic production of viral antigens may cause inflammation and necrosis, leading to elevation of liver enzymes from the necrotic hepatocytes, hepatitis, cirrhosis, hepatocellular carcinoma, and liver failure.

The HBV virion contains a compact, circular, partially double-stranded DNA genome that is 3.2 kb in length.1 It contains four overlapping open reading frames that code for seven proteins: the precore protein (also known serologically as e antigen or HBeAg) encoded on one transcript, the core protein (HBcAg) and the viral polymerase that are encoded in the same transcript, three forms of the envelope protein known as S antigen (HBsAg) encoded in three transcripts, and the X protein encoded on an independent transcript. Some chronic HBV carriers do not produce HBeAg (HBeAg−), typically due to mutations in the precore open reading frame or the precore promoter. All transcripts use the same polyadenylation signal. Viral DNA is synthesized by reverse transcription of the 3.5 kb RNA, which is terminally redundant and transcription of more than one genome length. The viral DNA is encapsidated by core proteins and enveloped with S proteins and membrane lipids from the host as the viral particles, also called Dane particles, bud from the endoplasmic reticulum of the hepatocyte and are secreted into the blood. The partially double-stranded viral DNA can be secreted in Dane particles to infect other hepatocytes or can be transported back to the nucleus by the viral nucleocapsid within the infected cell. Once in the nucleus, endogenous repair enzymes form a fully double-stranded, circular, and supercoiled form called ccc-DNA. This serves as the template for transcription of HBV genes and of the pregenomic RNA. It can remain in hepatocytes for years.

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Figure 1 Efficacy and safety of NAG-MLP delivery of chol-siRNA in mice. (a,b) ICR mice were injected intravenously with NAG-MLP at 1–16 mg/kg and 2 mg/kg chol-siRNA targeting either mouse F7 or luciferase as a control \((n = 4)\). After 48 hours, serum and plasma were collected and RNA was isolated from the liver. (a) F7 mRNA was measured relative to endogenous \(\beta\)-actin mRNA, open bars; and F7 activity was measured in serum, diagonally striped bars. F7 activity and mRNA were normalized to that in mice injected with isotonic glucose (IG). (b) Prothrombin time was measured in plasma. (c) Cholesterol-siRNA dose titration: ICR mice were injected intravenously with 6 mg/kg NAG-MLP or with 6 mg/kg of MLP with polyethylene glycol (PEG) attached instead of NAG (PEG-MLP); \(n = 4\). Chol-siRNAs targeting either mouse F7 (0.003–1 mg/kg) or luciferase (1 mg/kg) were coinjected with NAG-MLP. One group received 10 mg/kg chol-siF7 alone. F7 activity in serum was measured 48 hours later and was normalized to that in mice injected with isotonic glucose. (d,e) SV129 mice were injected four times with 6 mg/kg NAG-MLP and 5 mg/kg chol-siF7 \((n = 4)\). After 48 hours, serum and plasma were collected and RNA was isolated from the liver. (d) F7 activity and (e) clinical chemistries in serum were measured 24 and/or 48 hours after each injection. ALT, alanine transaminase; AST, aspartate transaminase; ALKP, alkaline phosphatase; BUN, blood urea nitrogen; Inj, Injection. Data bars indicate mean ± SD.

Titers of Dane particles vary greatly in infected patients and can be as high as \(10^{12}/\text{ml}\) in blood. Patients also secrete particles composed of HBsAg without the viral DNA. These subviral particles can be produced at levels as high as \(10^{12}/\text{ml}\) and up to 1,000 times more than the number of Dane particles. The highly abundant secreted HBsAg is believed to absorb virus-neutralizing antibodies, allowing the virus to spread and to be maintained in the host.¹

The current standard of care for treatment of chronic HBV infection is a daily oral dose of nucleotide/nucleoside analogs or a regimen of interferon or PEGylated interferon injections 1–7 times weekly for a year. In approximately 30% of HBeAg-positive (HBeAg⁺) carriers, the most effective of the current therapies may eventually lead to HBeAg seroconversion, loss of HBeAg expression together with production of anti-HBe antibodies. HBeAg seroconversion can result in an improvement of symptoms and a somewhat improved prognosis with regard to cirrhosis and hepatocellular carcinoma.² Nucleotide/nucleoside analogs effectively reduce viral DNA production by inhibiting the viral polymerase. Patients usually need to take these chemotherapeutics for life because viral replication quickly rebounds upon cessation of treatment. A more desirable outcome would be HBsAg seroconversion. An effective antibody response to HBsAg leads to clearance of the virus and a functional cure.³ Unfortunately, fewer than 10% of HBeAg⁺ patients experience HBsAg loss during treatment and no nucleotide/nucleoside analog treatment has been shown to significantly reduce HBsAg expression in HBeAg⁻ patients, which often have more severe disease.⁴⁻⁶
RNAi Therapeutics for Chronic HBV Infection

HBsAg clearance is similarly low from interferon treatment and these therapeutics cause significant side effects, including severe flu-like symptoms, marrow suppression, and autoimmune disorders. The lack of satisfactory therapeutics for treatment of chronic HBV infection highlights the need to approach this disease in a fundamentally different way.

RNA interference (RNAi)-based therapeutics have the potential to specifically knock down expression of viral proteins, including HBsAg, and viral replication. A major hurdle to the use of RNAi as a therapeutic for the treatment of chronic HBV infection has been the lack of safe and effective delivery of the RNAi trigger molecule. Other groups have used liposome particles or PEGylated nanoparticles to deliver siRNA targeting HBV, but with limited success. We previously described a polymer-based system, named Dynamic PolyConjugate (DPC), for the targeted delivery of siRNA to hepatocytes. Features of this system include an amphipathic, endosomolytic polymer that is reversibly masked in order that the membrane activity of the polymer is only revealed in the acidic environment of the endosome. Attachment of the targeting ligand, N-acetylgalactosamine (NAG), to the masking agent imparted hepatocyte-specific delivery via the highly expressed asialoglycoprotein receptor present on hepatocytes. Together, these features of the DPC allow highly efficient endosomal escape and cytoplasmic delivery of siRNA without toxicity associated with interaction with nontargeted cells and membranes.

In the original DPC formulation, the siRNA was covalently attached to the polymer via a bio-cleavable disulfide linkage. More recently, we have described a formulation in which the targeted and reversibly masked polymer is simply co-injected with a liver-tropic siRNA conjugated to cholesterol (chol-siRNA), eliminating the need for attachment to the polymer as in the prototypical DPC. This formulation resulted in a 500-fold improvement in the efficacy of chol-siRNA compared with injection of chol-siRNA alone. Removing the constraint of siRNA attachment allowed us to explore the use of other amphipathic polymers for chol-siRNA delivery. One of these amphipathic polymers is a peptide component of bee venom named melittin, which we had previously shown could be reversibly masked and used as an endosomolytic agent. In the present report, we show in proof-of-concept studies that coinjection of a melittin-like peptide (MLP) targeted to hepatocytes by the NAG ligand (NAG-MLP) with chol-siRNA is highly effective for knocking down the expression of an endogenous target gene, coagulation factor VII (F7), without toxicity in mice and nonhuman primates (NHPs). We go on to show that coinjection of NAG-MLP with chol-siRNAs targeting conserved HBV gene sequences results in multilog reduction of viral gene expression and viral DNA titer with long duration of effect in mouse models of chronic HBV infection. The use of NAG-MLP to facilitate chol-siRNA delivery should prove useful in the development of novel therapeutics for the treatment of chronic HBV infection in humans.

**RESULTS**

**Safety and efficacy of NAG-MLP for delivery of chol-siRNA in rodents and nonhuman primates**

We first evaluated efficacy of coinjecting NAG-MLP and chol-siRNA in mice using as a test target gene F7, which is expressed in hepatocytes and secreted into the bloodstream. The expression of F7 protein is easily measured using a simple serum-based assay. Cholesterol-conjugated F7 siRNA (chol-siF7) was coinjected with NAG-MLP intravenously (IV) into mice. Chol-siRNA targeting firefly luciferase (chol-siLuc) was coinjected with NAG-MLP as control. Using a constant dose of 2 mg/kg chol-siF7, the NAG-MLP dose was varied from 1 to 16 mg/kg and the effect on F7 mRNA and protein activity levels, as well as prothrombin time (PT), were measured 48 hours after injection (Figure 1a,b). Increasing the dose of NAG-MLP is expected to increase the efficiency of endosomal release of chol-siF7, enabling greater target gene knockdown. Knockdown of F7 mRNA in the liver was NAG-MLP dose dependent with approximately 80% knockdown in mice receiving 4 mg/kg NAG-MLP. No decrease in F7 mRNA expression was observed in animals coinjected with NAG-MLP and chol-siLuc. Reduction of serum F7 protein activity was also NAG-MLP dose dependent with a slightly steeper dose-response curve than that observed for F7 mRNA knockdown. Approximately 80% inhibition of serum F7 protein activity was achieved at a dose of 2 mg/kg NAG-MLP. Still greater levels of inhibition of F7 protein activity were achieved at higher doses of NAG-MLP, reaching 99% at 8 mg/kg NAG-MLP. As expected for decreased F7 protein activity in the blood, PT was significantly increased, beginning in mice receiving 4 mg/kg NAG-MLP. No effect on PT was observed in animals receiving NAG-MLP with the control chol-siLuc. Clinical chemistry analysis of these mice showed no elevation in liver enzymes (ALT, AST, and ALKP) nor in blood urea nitrogen, a marker for kidney toxicity, even in animals receiving the highest dose of NAG-MLP (Supplementary Figure S1).

Target gene knockdown was also responsive to the dose of chol-siRNA (Figure 1c). In this experiment, we used a constant dose of 6 mg/kg NAG-MLP as results from the previous experiment indicated that this dose is saturating, or nearly so, for endosomal release. In the presence of an IV injected dose of 6 mg/kg NAG-MLP (NAG-targeted), 0.01 mg/kg chol-siF7 knocked F7 activity down by approximately 50% in the mouse. A dose of 0.1 mg/kg chol-siF7 knocked F7 activity down 97%. Activity of the chol-siRNA and MLP required attachment of the liver targeting ligand (NAG) to the MLP. A dose of 1 mg/kg chol-siF7 with untargeted polymer (PEG-MLP) did not reduce the F7 target gene activity. There was also no significant knockdown from 10 mg/kg chol-siF7 in the absence of NAG-MLP (P = 0.09; Figure 1c). These results illustrate the tremendous increase in efficiency of chol-siF7 when it is coinjected with NAG-MLP. This is likely due to increased endosomal escape of the siRNA facilitated by the inclusion of a membrane active polymer in the injection as previously described.

Coinjection of chol-siRNA and NAG-MLP was then evaluated for efficacy and safety upon repeat-dosing of SV129 mice, the parental strain of HBV1.3.32 transgenic mice (Figure 1d,e). Mice were injected four times over the course of 5 weeks with NAG-MLP (6 mg/kg) and chol-siF7 (5 mg/kg) and evaluated at the indicated times for F7 expression and for markers of liver and kidney toxicity. As expected in mice receiving this dose level, F7 activity was reduced by 99% throughout the course of the experiment (Figure 1d). No elevations of liver enzymes or of kidney markers blood urea nitrogen and creatinine were observed in evaluations after each dose, indicating there is no cumulative toxicity (Figure 1e and data not shown).
To determine the efficacy of NAG-MLP in NHPs, cynomolgus monkeys were given an IV coinjection of 2 mg/kg chol-siF7 and 1 or 3 mg/kg NAG-MLP (Figure 2). Plasma F7 activity was knocked down 30–60% in monkeys coinfected with 1 mg/kg NAG-MLP and 2 mg/kg chol-siF7, and 97–99% in monkeys coinfected with 3 mg/kg NAG-MLP and 2 mg/kg chol-siF7 (Figure 2a). Plasma F7 activity was reduced at least 80% for approximately 1 month. As expected, the decreased F7 activity corresponded with an increase in clotting time (Figure 2b). F7 levels were unchanged from pre-injection at all dose levels of NAG-MLP. Together, these results indicate that coinfusion of NAG-MLP and chol-siRNA results in remarkably efficient target gene knockdown in mice and nonhuman primates without toxicity.

**Selection of siRNAs targeting HBV**

Having established a robust method for delivery of chol-siRNA to liver hepatocytes, we investigated whether this method could be applicable to the treatment of chronic HBV infection. Our selection process for identifying lead siRNAs to target HBV began with in silico methods to determine sequences that are highly conserved among known HBV genomes. An in silico specificity filter then deselected those with close sequence similarity to mRNAs of the human and mouse transcriptomes (NCBI RefSeq release 41). The selected candidate siRNAs were then tested in cell culture by in vitro transfection experiments for mRNA silencing activity to rank the siRNAs for potency.

In the first step of the siRNA selection process, highly conserved sequences in the HBV genome were identified by computational analysis. Canonical siRNAs are based on 19 nucleotides of complementary sequence, but mismatches at the terminal ends are generally well tolerated and have much less effect on the efficacy of the siRNA than internal mismatches. Therefore, an initial set of 2,754 full-length human HBV genome sequences of genotypes A–D (NCBI GenBank) was analyzed for 17-nucleotide stretches that were identical in at least 90% of these genomes. In the second step, sequences that would cross-react with human genes were deselected and sequences containing four or more consecutive guanidine bases were excluded (data not shown). This analysis identified 140 highly conserved 17-mer sequences that could potentially be siRNA target sites. In the third step, the most conserved 19-mer sequences that contained these 17-mers in positions 2–18 were identified. This selection resulted in 140 candidate 19-mer siRNA sequences (Figure 3a). These tended to be in regions of overlapping reading frames. Due to the structure of the HBV genome, these sequences are almost always located in the overlapping reading frame.
None of the 140 conserved 17-mer sequences matched 100% of the initial 2,754 HBV sequences (data not shown). The most highly conserved sequence was identical to 98.5% of the genome sequences. When two of these highly conserved siRNAs were evaluated together; however, the combination included a perfect match for 99.6–99.9% of all these HBV genomes (data not shown).

The 140 candidate siRNAs were synthesized and screened in Cos-7 green monkey kidney cells using a plasmid containing Renilla and firefly luciferase genes (Figure 3b). Target sites for all the HBV siRNAs were in the 3′ untranslated region of the Renilla luciferase gene in psiCHECK-HBV. At 10 nmol/l, 46 siRNAs gave ≥50% knockdown and 14 gave ≥75% knockdown (Figure 3b).

Heavy chemical modification of siRNAs protects them from rapid degradation in serum and also prevents cytokine induction and off-target effects. Therefore, the 23 most effective siRNAs were synthesized with 2′-O-methyl/2′-fluoro-phosphoramidites and screened using the same in vitro assay (data not shown). The four most potent siRNAs performed well in both screens, as lightly or heavily modified siRNAs. They were named siHBV-74, siHBV-75, siHBV-76, and siHBV-77 (see locations in Figure 3a). The target sites of the four most effective siRNAs are shown in Table 1.

Bases 2–18 of the four siHBV lead sequences were analyzed for their conservation in genotypes A–H as annotated in the NCBI genome with overlapping mRNAs, a single siRNA would have the ability to target multiple viral RNAs.

Table 1 Target sites for HBV siRNAs

| siRNA ID | Sequence (5′-3′) | Position | 3.5 kb | 2.4 kb | 2.1 kb | 0.7 kb |
|----------|-----------------|----------|--------|--------|--------|--------|
| siHBV-75 | UCUGCGCGGUUUUAAUC | 380–398 | +      | +      | +      | −      |
| siHBV-76 | UUUACUAGUGCCAUUUUGUU | 674–692 | +      | +      | +      | −      |
| siHBV-74 | CUGUAGGCAUAAAUUGGUC | 1,779–1,797 | +      | +      | +      | +      |
| siHBV-77 | ACCUCUGGCCUAAUCACUC | 1,825–1,843 | +      | +      | +      | +      |

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Bases 2–18 of the four siHBV lead sequences were analyzed for their conservation in genotypes A–H as annotated in the NCBI genome with overlapping mRNAs, a single siRNA would have the ability to target multiple viral RNAs.
modified siHBVs (chol-siHBV) were synthesized and coinjected in vitro for AG-MLP–delivered HBV siRNA delivery in all genotypes.

Table 2 Conservation of candidate HBV siRNAs was evaluated by genotype, using the 17-mer sequences from positions 2–18. Shown are the percentages of sequences from each genotype that contain the identical sequence to each siRNA (Full) or that have up to one mismatch (Full + 1). Only sequences with annotated genotypes as described in NCBI GenBank were analyzed.

| siRNA ID | Genotype A (n = 303) | Genotype B (n = 375) | Genotype C (n = 827) | Genotype D (n = 350) | Genotype E (n = 181) | Genotype F (n = 60) | Genotype G (n = 9) | Genotype H (n = 12) |
|----------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| siHBV-75 | 95.5 99.8 96.0 99.5 95.5 99.8 96.0 97.7 98.9 99.4 98.3 100 100 100 100 100 |
| siHBV-76 | 94.0 97.8 94.7 98.4 94.0 97.8 98.0 99.7 100 100 100 100 100 100 100 100 |
| siHBV-77 | 96.4 98.9 91.7 98.7 96.4 98.9 91.7 96.3 99.4 99.4 96.7 100 100 100 100 100 |

*Conservation of candidate HBV siRNAs was evaluated by genotype, using the 17-mer sequences from positions 2–18. Shown are the percentages of sequences from each genotype that contain the identical sequence to each siRNA (Full) or that have up to one mismatch (Full + 1). Only sequences with annotated genotypes as described in NCBI GenBank were analyzed.

GenBank database (Table 2). These were highly conserved across all genotypes.

**In vivo efficacy of NAG-MLP–delivered HBV siRNAs on HBV infection**

For in vivo evaluation of the four lead siHBVs identified in the in vitro screen, cholesterol-conjugated versions of the chemically modified siHBVs (chol-siHBV) were synthesized and coinjected with NAG-MLP. We used two murine models of chronic HBV infection in these studies. Both models use a terminally redundant full-length human HBV genome (HBV1.3). In the first model, the HBV1.3 sequence is encoded in a plasmid (pHBV1.3), which is then delivered to the liver of the mouse by hydrodynamic tail vein (HTV) injection. This technique delivers the plasmid DNA almost exclusively to hepatocytes in the liver, typically transfecting 5–20% of the hepatocytes. Expression of the HBV...
genome from the plasmid results in long-term production of the viral mRNAs and pregenomic RNA used to support viral replication and the production of viral proteins and HBV virions. The immune-deficient NOD-SCID mouse was used for these studies. This strain does not produce antibodies to HBV, but any damage to the liver can result in loss of the pHBV1.3 by either cell death or cell division. Therefore, mice were allowed at least 3 weeks following HTV injection to ensure HBsAg expression from the pHBV1.3 had stabilized and that mice had fully recovered from any liver damage that may have been caused by the hydrodynamic injection. We refer to this as the pHBV model. The second model is the transgenic HBV1.3.32 mouse that has one chromosomally integrated copy of the over-length HBV1.3 genome and allows HBV gene expression and replication as in the pHBV model. In this case, the HBV genome is in every hepatocyte, a scenario that resembles the infected patient. However, measurement of HBsAg in serum is not meaningful in this mouse strain because most of the HBV1.3.32 mice in the SV129 background develop antibodies to HBsAg (data not shown).

**In vivo efficacy and duration of effect of chol-siHBV in a pHBV mouse model of chronic infection**

To determine the relative efficacy of the four chol-siHBVs in *vivo*, each was coinjected with NAG-MLP into the pHBV mouse model. In the first experiment, each mouse received an IV dose of NAG-MLP at 6 mg/kg together with one of the chol-siHBVs at a relatively low dose of 0.25 mg/kg. We chose this dose of NAG-MLP as it was shown to be saturating for endosomal release as measured by efficacy in the mouse F7 knockdown studies (Figure 1a).

HBsAg levels in serum and the number of HBV genome copies in DNA isolated from the serum were monitored until expression levels in the siRNA-treated groups returned to levels similar to those in a control group injected with just isotonic glucose (Figure 4a and data not shown). Maximal knockdown of 1.8 ± 0.1 log₁₀ (average 61-fold) was obtained from chol-siHBV-74 and occurred 7 days after siRNA delivery (day 8 in Figure 4a). The second most effective siRNA was chol-siHBV-77, yielding 1.4 ± 0.1 log₁₀ knockdown (average 27-fold), also at day 8. Maximal knockdown from chol-siHBV-75 (1.1 ± 0.1 log₁₀) and chol-siHBV-76 (0.9 ± 0.1 log₁₀) occurred on day 4.

The dose response and duration of effect of the chol-siHBV siRNAs was evaluated by IV coinjection of a higher dose of chol-siHBV (6 mg/kg) and the same dose of NAG-MLP (6 mg/kg) used in the previous experiment (Figure 4b). Significantly higher levels of HBsAg knockdown and longer durations of effect were observed for all chol-siHBVs injected at this dose than at the 0.25 mg/kg dose level. Maximal knockdown of HBsAg in mice receiving NAG-MLP and chol-siHBV-74 was 3.2 ± 0.1 log₁₀ (>1,000-fold), to the limit of detection, and occurred 3 weeks after siRNA delivery. HBsAg was reduced at least 2 log₁₀ (100-fold or ≥99% knockdown) for 1 month following a single coinjection. Knockdown in mice coinjected with NAG-MLP and chol-siHBV-77 was more than 3 log₁₀ (>1,000-fold) from day 8 until day 22, though the RNAi effect began to subside more quickly in these mice than in those receiving chol-siHBV-74. Maximal knockdown in mice coinjected with NAG-MLP and 6 mg/kg of the chol-siHBV-75 (2.9 ± 0.1 log₁₀) or chol-siHBV-76 (3.1 ± 0.7 log₁₀) occurred at day 8 and was marginally less than that observed using chol-siHBV-74 or chol-siHBV-77, consistent with the results obtained at lower doses of the four chol-siHBVs as shown in Figure 4a. The duration of effect observed using chol-siHBV-75 and chol-siHBV-76 was also more limited. No significant HBsAg knockdown was observed in mice injected with NAG-MLP alone, chol-siHBVs alone, or chol-siF7p (primate F7) coinjected with NAG-MLP (Supplementary Figure S2a). The dose of 6 mg/kg chol-siHBV is likely saturating as the difference in knockdown in mice receiving 3 mg/kg chol-siHBV with 6 mg/kg NAG-MLP is minimal in terms of magnitude and duration of effect (data not shown). A lower dose of NAG-MLP does, however, reduce efficacy from 3 mg/kg chol-siHBVs (Supplementary Figure S2a). Evaluation of liver enzymes at 5, 10, or 15 weeks after injection of NAG-MLP and chol-siHBVs showed no significant differences in ALT or AST compared with uninject ed or isotonic glucose-injected pHBV mouse controls, indicating that there is no delayed toxicity (Supplementary Figure S2b).

HBcAg levels were also evaluated in pHBV mice that had been coinjected with 6 mg/kg NAG-MLP and 6 mg/kg chol-siHBV-74, -75, -76, or -77 (Figure 4c). Coinjection with chol-siHBV-74, -76, or -77 resulted in knockdown of HBcAg levels below the limit of detection of the assay at 24 hours after siRNA delivery. Maximal knockdown from chol-siHBV-75 was 85%. Recovery of HBcAg within the time frame of this experiment was only observed in mice receiving chol-siHBV-75 or 76, consistent with the shorter duration of HBsAg knockdown in mice receiving these chol-siHBVs.

The effect of chol-siHBV delivery on production of viral particles was determined by collecting serum from pHBV mice during the course of a 50-day RNAi study and quantitating by qPCR the number of HBV genome copies in pooled serum samples (Figure 4d). Mice were coinjected with 6 mg/kg NAG-MLP and 6 mg/kg chol-siHBV-74, -75, -76, or -77. Reduction in the number of copies HBV DNA/ml serum followed a time course very similar to that of HBsAg knockdown (compare Figure 4d, b). Maximal reduction of viral DNA following delivery of chol-siHBV-74 (2.4 log₁₀, 250-fold) and chol-siHBV-77 (2.6 log₁₀, 400-fold) occurred on day 22. Viral DNA in serum was reduced more than 90% for 1 month following a single chol-siHBV-74 delivery. Together, these results indicate that the four siHBVs shown to be most efficacious when transfected *in vitro* were all highly effective when coinjected with NAG-MLP *in vivo*, dramatically reducing viral serum proteins HBsAg and HBcAg and viral DNA levels. Of the four chol-siHBV sequences tested, chol-siHBV-74 and chol-siHBV-77 were the most efficacious in terms of both magnitude of knockdown and duration of effect by all of these measures.

The effect of coinjection of NAG-MLP and chol-siHBV-74 or chol-siHBV-77 on the level of viral transcripts was also investigated. HBV produces at least 6 mRNA species. These are 3.5 kb (two types), 2.4 kb, 2.1 kb (two types), and 0.7 kb. The precore and core open reading frames of the HBV genome encode overlapping in-frame proteins that are translated from two separate 3.5 kb mRNAs. The 3.5 kb mRNA that encodes HBcAg is transcribed from the precore promoter located approximately 30 bases upstream of the core promoter. The other 3.5 kb mRNA is the pregenomic RNA; it is translated to produce core antigen (HBcAg) and the polymerase, in addition to being reverse transcribed to...
generate the virion DNA. The 2.4 kb mRNA that codes for the large HBsAg polypeptide is a minor species. The 2.1 kb RNAs code for the middle and major HBsAg polypeptides. The 0.7 kb mRNA that codes for X protein is usually undetectable.

To determine the efficacy of chol-siHBV-74 and chol-siHBV-77 for knockdown of viral transcripts, these chol-siHBVs were coinjected with NAG-MLP into pHBV mice. In this experiment, the mice received 6 mg/kg NAG-MLP and either 1 or 6 mg/kg chol-siRNA-74 or -77. The qPCR probes used in this experiment were designed to hybridize with both types of 3.5 kb mRNAs, the 2.4 kb mRNA and the 2.1 kb mRNAs. Thus, this assay would give a measure of the total amount of these viral RNAs in the liver.

Coinjection of either of these two chol-siHBVs with NAG-MLP reduced viral RNA content in liver. Two weeks after coinjection, chol-siHBV-74 and -77 reduced the levels of measurable HBV RNA by 87% and 91%, respectively, at 1 mg/kg and by 92% (1.1 log10) and 95% (1.3 log10), respectively, at 6 mg/kg (Figure 4e).

No effects on viral RNA levels in liver were observed in animals receiving the control chol-siLuc together with NAG-MLP.

Clinical chemistry analysis of serum collected from pHBV mice 24 hours after NAG-MLP and chol-siRNA injection showed no elevations in liver enzymes (ALT, AST) or in blood urea nitrogen.
However, HBeAg levels in serum were substantially decreased, which of the two 3.5 kb viral mRNAs persists using our methods. Reduced (data not shown). These studies demonstrate that chol-liver were visualized by northern blotting (Figure 5a). Consistent were killed 7 days after coinjection (day 8) and HBV RNAs in the effect on the 2.1 kb RNA was similar to HBsAg results in the pHBV combined chol-siHBV-74+77 (data not shown). As stated previ-
sal decrease in the levels of the 3.5 kb HBV RNAs (this mouse strain. by this mRNA due to the presence of anti-HBsAg antibodies in
reduced the level of the 3.5 kb RNA by 87% (data not shown). These results indicate chol-siHBV-74 and chol-
siRNAs targeting two highly conserved sequences would decrease the probability of the emergence of viral resistance in a clinical setting. HBV1.3.32 mice were coinjected with 6 mg/kg NAG-MLP and chol-siHBV-74 and chol-siHBV-77 in combination (chol-
RNA and DNA 1 week after the second injection (Figure 5c–e). Consistent with data obtained from mice receiving a single dose (Figure 5a), northern blot analysis revealed near elimination of the 2.1 kb RNA after two weekly coinjections of NAG-MLP and chol-siHBVs separately or in combination, compared with control mice (Figure 5c). Knockdown of the 3.5 kb RNA was also greatly reduced as determined by northern blot and by reverse transcription qPCR, with knockdown levels very similar to those following a single coinjection (Figure 5c,d). Analysis of the replicative intermediates by Southern blot indicated that all were reduced to the limit of detection in mice coinjected with NAG-MLP and chol-siHBV-74, chol-siHBV-77, or chol-siHBV-74+77 (Figure 5e). Further evidence of reduced viral replication was obtained by immunohistochemical analysis of HbcAg, a marker of viral replication, in liver sections (Figure 5f). Two weekly coinjec-
tions of NAG-MLP and chol-siHBV-74+77 greatly reduced expression of HbcAg in the cytoplasm of the hepatocytes compared with control mice injected with either isotonic glucose or coinjected with NAG-MLP and the control chol-siLuc (Figure 5f). These results suggest that not only does coinjection of NAG-MLP and the chol-siHBVs dramatically decrease viral protein produc-
tion, but also it strongly inhibits viral replication.
Safety assessments confirmed that coinjection of NAG-MLP and chol-siHBVs was well tolerated in these mice. Serum clinical chemistry was normal after injection (Supplementary Figure S3) and analysis of cytokine/chemokine levels using a panel of 25 showed no induction in these markers at 6 hours or 48 hours after injection (data not shown).

**DISCUSSION**

The general strategy of chol-siRNA coinjection with hepatocyte-targeted NAG-MLP represents a seminal advance in the field of siRNA delivery on several fronts. First, a dramatic increase in the efficacy of chol-siRNA is obtained using this method. Levels of knockdown in both mice and nonhuman primates reported in these studies are, to our knowledge, unprecedented. Second, the system is fully dependent on the presence of the NAG ligand on the endosomolytic agent MLP. Cell-specific targeting is desirable to improve efficacy and reduce the potential side effects. We presented evidence here that the presence of NAG on the MLP is required for knockdown of the hepatocyte-expressed gene encoding Factor 7. We have previously shown that the presence of an intact cognate receptor for NAG, the asialoglycoprotein receptor, is required for knockdown using NAG targeting of another endosomolytic polymer used in the coinjection scenario.12 Together, these data indicate that this delivery system is targeted to liver hepatocytes. Third, the MLP itself is a small peptide composed

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**Knockdown of HBV in a transgenic mouse model of chronic HBV infection**

The two most effective chol-siHBVs, chol-siHBV-74 and chol-
siHBV-77, were evaluated for their efficacy at reducing viral products in the transgenic HBV mouse model by coinjection with NAG-MLP (Figure 5). Although both of the lead siRNAs target highly conserved sequences, together they provide even greater coverage for the variety of HBV genomes in the infected population. Bases 2–18 of siHBV-74 are identical to 96.4% of 2,754 evaluated genome sequences and those of siHBV-77 are identical to 92.6% of the genomes. Together, their coverage is 99.64%. Use of siRNAs targeting two highly conserved sequences would decrease the probability of the emergence of viral resistance in a clinical setting. HBV1.3.32 mice were coinjected with 6 mg/kg NAG-MLP and chol-siHBV-74 and chol-siHBV-77 in combination (chol-
-80% knockdown 1 month after a single coinjection of the combined chol-siHBV-74+77 (data not shown). As stated previously, we are not able to measure serum levels of HBsAg produced by this mRNA due to the presence of anti-HBsAg antibodies in this mouse strain.

Inspection of the northern blot also revealed a substantial decrease in the levels of the 3.5 kb HBV RNAs (Figure 5a). RT-qPCR quantitation of the 3.5 kb HBV RNAs using a probe in the precore/core region indicated that coinjection of NAG-MLP and chol-siHBV-74+77 reduced the 3.5 kb RNA by 91% (P = 0.009) compared with control animals that received NAG-MLP and pri-
-80% knockdown 1 month after a single coinjection of the combined chol-siHBV-74+77 (data not shown). As stated previously, we are not able to measure serum levels of HBsAg produced by this mRNA due to the presence of anti-HBsAg antibodies in this mouse strain.

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of naturally occurring L-amino acids and, therefore, expected to be fully biodegradable. This feature is highly desirable and would allow multiple doses to be administered without the potential of toxicity due to its accumulation. Our preliminary studies indicate that >95% of the NAG-MLP in the liver is degraded by 24 hours after dosing and that multiple doses are well tolerated in rodents and nonhuman primates (here and data not shown). Importantly, generation of anti-MLP antibodies in these multidose studies was not detected (data not shown). Finally, the formulation is simple, requiring simple coinjection of the NAG-MLP and chol-siRNA without requiring preformation of a siRNA/delivery vehicle complex. This reduces complexity and would facilitate ease in manufacturing.

We have shown that high-level knockdown can be achieved in mice and in nonhuman primates using this siRNA delivery system. For use in humans, it is important to consider the dose levels that will be required for the desired knockdown level using this system. Although this cannot be precisely known until tested in clinical trials, the animal data generated in this report can be used as a predictor. The amount of chol-siF7 required for maximal knockdown when coinjected with NAG-MLP is 2 mg/kg in both mice and NHPs (Figure 1a and data not shown). As the chol-siF7 dose required for maximal knockdown according to this data appears to be species independent, we anticipate the doses on a mg/kg basis of chol-siRNA required for maximal efficacy will be the same, or nearly the same, in humans as in our test species. The dose of NAG-MLP required for >95% F7 activity knockdown is approximately twofold lower in NHPs than in mice on a mg/kg basis (compare Figures 1a and 2a). As for chol-siRNA, there does not appear to be a penalty in the dose required in larger animals. These data suggest that, to a first approximation, the dose level shown to be efficacious in the animal studies will translate to humans on a mg/kg basis.

The potential use of RNAi to treat chronic hepatitis B infection has been previously explored using a variety of approaches.\textsuperscript{8,9,28–30} The simplicity of the siRNA delivery system described here and its targetability to liver hepatocytes is unique and positions it as a potential therapeutic for the treatment of diseases associated with the liver, including Hepatitis B, for which novel therapeutics are needed. We found that coinjection of NAG-MLP and chol-siHBV in mouse models of HBV infection results in profound reductions in the levels of HBV mRNAs, proteins, and viral DNA, with long duration of effect. This duration of effect after a single dose suggests that a monthly dosing regimen would be sufficient for use in clinical settings. A longer duration of effect has only been reported in HBV mice receiving adeno-associated virus expressing a short hairpin RNA (shRNA) targeting HBV.\textsuperscript{36} Although this vector-based approach is promising, lingering concerns over the long-term effects of constitutive shRNA expression remain.\textsuperscript{31}

Reduction of viral antigens using RNAi is especially intriguing as researchers have for some time postulated that reduction of viral antigens may allow awakening of an immune response that leads to a functional cure. The cytotoxic T lymphocyte response is vigorous and polyclonal in acute HBV infection, but weak during chronic infection.\textsuperscript{32,33} Even so, HBV-specific cytotoxic T lymphocytes have been found at low levels in the liver and in the periphery of chronically infected patients, including T lymphocytes that recognize the HBV envelope proteins (HBsAg).\textsuperscript{34,35} These findings have led to the speculation that the HBsAg-specific T cells may be inhibited by the high HBsAg production.\textsuperscript{36} Expansion of the small HBV-reactive cytotoxic T lymphocyte population would allow a chronically infected patient to mount an immune response more similar to that of an acutely infected patient who is able to clear viral infection. RNAi suppression of HBsAg production as shown in our study specifically would allow the expansion of such HBsAg-specific cytotoxic T lymphocytes and facilitate HBsAg seroconversion. Recent studies of patients on HBV therapeutics have shown that a tenfold HBsAg reduction during nucleotide/nucleoside analog or interferon treatment is highly predictive of patients who will seroconvert for HBsAg and be functionally cured.\textsuperscript{37–39} The percentage of patients with such a sustained response from current therapeutics is low. An effective RNAi therapeutic may provide a much greater opportunity for immune response awakening than can be obtained with other treatment modalities.

In this study, we presented data on the efficacy of a new siRNA delivery system and showed its utility in wild-type mice, nonhuman primates, and in mouse models of HBV infection. Coinjection of this hepatocyte-targeted, endosomolytic polymer and chol-siHBVs would be a fundamentally distinct therapeutic for treatment of chronic HBV infection and holds great promise to become a functional cure for patients.

**MATERIALS AND METHODS**

**Plasmids.** The Dual-Luciferase HBV reporter plasmid psiCHECK-HBV, containing a synthetic version of the *Renilla* luciferase (hRluc) gene and a synthetic firefly luciferase gene (hLuc'), was constructed to test activity of HBV siRNAs. HBV sequence regions 84–805, 1,075–1,992, 2,165–2,530, and 2,718–2,940 of GenBank accession #EU554538.1 (genotype C) were joined in *silico*, restriction sites were added at either end, and this sequence was then chemically synthesized by Geneart (Regensburg, Germany) and cloned into the Xhol/NotI site of psiCHECK-2 Dual-Luciferase vector (Promega, Madison, WI).

Plasmid pHBV1.3 contains the same terminally redundant HBV1.3 sequence as in the HBV1.3.32 transgenic mice (GenBank accession #V01460).\textsuperscript{19} HBV1.3 was synthetically constructed (DNA2.0, Menlo Park, CA) and inserted into pUC-based cloning vectors (ampicillin or kanamycin resistant), which were both used, but only one type of plasmid in each study. MC-HBV1.3 is a minicircle derived from pHBV1.3 by recombination that removes all the bacterial sequence and was prepared by PlasmidFactory (Bielefeld, Germany).\textsuperscript{40} HBsAg expression from this construct is higher than from pHBV1.3.

Two plasmids were used as assay controls, pHCR/Ubc-SEAP and pSEAP-HBV353–777. Plasmid pHCR/Ubc-SEAP contains the human ApoE hepatic control region, the human ubiquitin C promoter, and human placental secreted alkaline phosphatase gene.\textsuperscript{41} It was spiked into mouse serum as a recovery control for the purification of HBV DNA particles in serum. Plasmid pSEAP-HBV353–777 was used to generate a standard curve for the quantitative real-time PCR (qPCR) assay to determine the number of copies of HBV in serum samples. This plasmid contained a segment of HBV sequence within the S gene, bases 535–777 of GenBank accession #V01460.

**Animals and procedures.** All animal studies were carried out in accordance with the National Research Council’s *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Wisconsin–Madison, the University of Illinois–Chicago and or the Arrowhead Madison Animal Care and Use Committees.

Female ICR, C57BL/6, and NOD.CB17-Prkdc<sup>S<sub>−/−</sub></sup>/NcrCrl (NOD-SCID) mice, 6–8 weeks old, were obtained from Harlan Sprague-Dawley
The production and characterization of the HBV transgenic mouse lineages 1.3.2 and 1.3.3 has been described.19 These HBV transgenic mice contain a single copy of the terminally redundant, 1.3-genome length copy of the HBV genome (GenBank accession #V01460) integrated into the mouse chromosomal DNA. High levels of HBV replication occur in the livers of these mice. The mice used in the breeding experiments were homozygous for the HBV transgene and were maintained on the SV129 genetic background.42 Hemizygous HBV transgenic mice were generated by mating the HBV transgenic mice with SV129 mice. The resulting HBV transgenic F1 mice were used for all subsequently analysis. Male and female mice were used, but each experiment contained mice of a single gender.

Cynomolgus monkeys were 4- to 5-year-old males weighing 4–5 kg and were housed at the University of Wisconsin–Madison. Before injection, animals were sedated with Ketamine (5 mg/kg by intramuscular injection (IM)) and Dexdomitor (0.015 mg/kg, IM). All NAG-MLP/chol-siRNA injections were into the small saphenous vein over a 1- to 2-minute period. After this injection, monkeys were given the Dexdomitor reversal drug Antisedan (0.15 mg/kg, IM).

For siRNA delivery, a mixture of chol-siRNA, NAG-MLP, and Heps-buffered (5 mmol/l, pH 7.5) isotonic glucose was delivered IV, typically in a volume of 200 µl per mouse and 8–10 ml in a cynomolgus monkey. Mice in control groups were injected with isotonic glucose alone.

The siRNAs were synthesized by Roche (Kulmbach, Germany) using standard phosphoramidite chemistry and contained the following sequences (5′-3′): chol-siHBV-74 sense, chol-uAuCfuGfuAfGfcAfufAfuUufGfuAfufAf(TmdT); chol-siHBV-74 antisense,dTufAfuUfuGfuAfufAfAfAfGfcAfufAfuUufGfuAf(TmdT); chol-siHBV-75 sense, chol-uAuUfuUfuGfuAfufGfAfufUfuGfuAf(TmdT); chol-siHBV-75 antisense, dTufAfuUfuGfuAfufAfAfAfGfcAfufAfuUufGfuAf(TmdT); chol-siHBV-76 sense, chol-uAuUfuUfuCfuAfAfAfGfcAfufAfuUufGfuAf(TmdT); chol-siHBV-76 antisense, dTufAfuUfuGfuAfufAfAfAfGfcAfufAfuUufGfuAf(TmdT); chol-siHBV-77 sense, chol-uAuAfuCfuAfAfAfGfcAfufAfuUufCfuAfAf(TmdT); chol-siHBV-77 antisense, dTufAfuUfuGfuAfufAfAfAfGfcAfufAfuUufGfuAf(TmdT); chol-siLuc sense, chol-uAuCfuUfuGfuAfAfAfAfGfcAfufAfuUufGfuAf(TmdT); chol-siLuc antisense, dTufAfuUfuGfuAfufAfAfAfGfcAfufAfuUufGfuAf(TmdT).

Serum factor VII and prothrombin time. Serum factor VII levels were measured by a chromogenic method according to the kit manufacturer’s instructions (Biophen FVII, Ref 221404, Hyphen BioMed, Neuville-sur-Oise, France) and using a microplate reader to measure absorbance at 405 nm. For mouse PT measurements, blood was collected and mixed with sodium citrate (0.109 mol/l) as an anticoagulant. For each sample, one part anticoagulant was mixed with nine parts whole blood. After mixing, blood was centrifuged for 3 minutes at 9,000 g to pellet cells. The plasma supernatant was used to measure PT with a mechanical clot detection system (KC1 Delta Coagulation Analyzer; Trinity Biotech, Bray, Ireland). For this test, plasma was warmed to 37°C in a cuvette with a small metal ball that mixes continuously. After a brief incubation, tissue factor with calcium (Thromboplastin D; Thermo Scientific, Middletown, VA) was added and a timer was started. The timer stopped automatically once the metal ball was displaced by the formation of the fibrin clot. PT analysis for NHP samples was performed by Meriter Laboratories (Madison, WI).
and frozen in small aliquots. The level of HBsAg expression in the serum was determined by using recombinant HBsAg protein, ayw subtype (Aldevron).

**Immunohistochemical analysis of HBV core antigen in liver.** Immunohistochemical detection of HBcAg in paraffin-embedded mouse liver sections was performed as previously described. Tissue samples were fixed in 10% zinc-buffered formalin, embedded in paraffin, sectioned (3 μm), and stained with hematoxylin as described elsewhere. The intracellular distribution of HBcAg was assessed by the labeled-avidin-biotin detection procedure exactly as described elsewhere. Briefly, paraffin-embedded sections in PBS, pH 7.4, were treated for 10 minutes at 37°C with 3% hydrogen peroxide and washed with PBS. After the sections were blocked with normal goat serum for 30 minutes at room temperature, rabbit anti-HBcAg (Dako, Carpinteria, CA) primary antisera was applied at a 1:100 dilution for 60 minutes at 37°C. After a wash with PBS, a secondary antiserum consisting of biotin-conjugated goat anti-rabbit immunoglobulin G F(ab)2 (Sigma, St. Louis, MO) was applied at a 1:100 dilution for 30 minutes at 37°C. The antibody-coated slides were washed with PBS, treated with the streptavidin-horseradish peroxidase conjugate (Extravidin; Sigma) at a 1:60 dilution for 30 minutes at 37°C, stained with 3-amino-9-ethyl carbazole (Shandon-Lipshaw, Pittsburgh, PA), and counterstained with Mayer's hematoxylin before being mounted.

**HBV DNA isolation from mouse serum.** Equal volumes of serum from mice in a group were pooled to a final volume of 100 μl. DNA was isolated from serum samples using the QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Sterile 0.9% saline was added to each sample to a final volume of 200 μl. Serum samples were added to tubes containing buffer and protease, according to the instructions, followed by addition of carrier RNA from the kit to aid in the isolation of small amounts of DNA. Each sample was processed using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Each assay requires 2–23 μl serum, depending on the test. For NHP samples, complete blood counts and clinical chemistries were evaluated by Meriter Laboratories. Cytokine levels were measured in serum using the MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel with premixed 25-plex (catalog #MCYTOMAG-70K-PMX, EMD; Millipore, Billerica, MA) or the primate cytokine 23-plex (catalog #MPXPRCYTO40P23; Millipore) using a Luminex 200 instrument (Invitrogen, Carlsbad, CA).

**Clinical chemistry and cytokine analysis.** Clinical chemistry markers in mouse serum were measured using a COBAS Integra 400 (Roche Diagnostics, Indianapolis, IN) chemical analyzer according to the manufacturer's instructions. Each assay requires 2–23 μl serum, depending on the test. For NHP samples, complete blood counts and clinical chemistries were evaluated by Meriter Laboratories. Cytokine levels were measured in serum using the MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel with premixed 25-plex (catalog #MCYTOMAG-70K-PMX, EMD; Millipore, Billerica, MA) or the primate cytokine 23-plex (catalog #MPXPRCYTO40P23; Millipore) using a Luminex 200 instrument (Invitrogen, Carlsbad, CA).

**DNA and RNA analysis.** The number of copies of HBV genomes in DNA isolated from the pHBV mouse model serum (undiluted) was determined by qPCR using a standard curve for absolute quantitation. Plasmid pSEAP-HBV353-777, encoding a short segment of the HBV genome within the S gene, was used to create a standard curve over a range of 6 logs (10–10 copies). Similarly, plasmid pHCR/UbC-SEAP was used as a standard curve (10–10 copies) to assess the general recovery of DNA from serum samples. TaqMan chemistry-based primers and probes with fluor (ZEN/IBFQ were used: primers 5′-GCGGAGCTGATGACTA-3′ and 5′-GG TACAGCAACAGGAGGGATACATA-3′, and 215 – 235 RF-labeled reporter 5′-CTGCTCAAGGAACTC-3′ for detection of HBV (Life Technologies, Grand Island, NY); and primers 5′-CATGCCACCTCCAACATCCACTC-3′ and 5′-GCCGGACCTGCATGACTA-3′, and 6-carboxyfluorescein (FAM)-labeled reporter 5′-CTGCTCAAGGAACTC-3′ for detection of HBV. Gene expression assays for HBV and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA to detect the GAPDH transcript used as an internal control.

QPCR following a reverse transcription step (RT-qPCR) was used to measure the level of GAPDH and HBV 3.5 kb transcripts in HBV1.3.32 mouse liver RNA. After DNase I treatment, 1 μg of RNA was used for cDNA synthesis using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA), followed by qPCR quantification using SYBR Green and an Applied Biosystems 7300 real-time thermocycler. Thermal cycling consisted of an initial denaturation step for 10 minutes at 95°C followed by 40 cycles of denaturation (15 seconds at 95°C) and annealing/extension (1 minute at 60°C). The relative HBV 3.5 kb RNA expression levels were estimated using the comparative C (ΔC) method with normalization to mouse GAPDH RNA. The PCR primers used were 5′-GCCCTTATCTCATACAAACCTCGCCG-3′ (HBV 3.5 kb RNA sense primer, coordinates 2,311–2,335), 5′-TTCGCTTGCCAGGCGAGGGA-3′ (HBV 3.5 kb RNA antisense primer, coordinates 2,401–2,382), 5′-TCTGG AAAAGTCGAGCGTG-3′ (mouse GAPDH sense primer), and 5′-CCA GTAGCTTCCCGTTACAG-3′ (mouse GAPDH antisense primer).

For liver RNA samples from pHBV mice, 50–500 ng of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The cDNA was then diluted 1:50 and multiplex RT-qPCR was performed using 5′ exonuclease (TaqMan) chemistry with forward primer 5′-GCCGACCTGTCCATGACTA-3′, reverse primer 5′-GGTACAGCAACAGGAGGGATACATA-3′, and 6-carboxyfluorescein (FAM)-labeled reporter 5′-CTGCTCAAGGAACTC-3′ for detection of HBV. Gene expression assays for HBV and mouse β-actin, and Gene Expression Master Mix (Life Technologies) were used. The data were analyzed using the ΔC, method of relative quantification.

**SUPPLEMENTARY MATERIAL**

**Figure S1.** Controls for NAG-MLP and chol-siRNA coinjection in the pHBV mouse model.

**Figure S2.** Controls for NAG-MLP and chol-siRNA coinjection in the pHBV mouse model.

**Figure S3.** Clinical chemistry in transgenic HBV1.3.32 mice.

**Table S1.** Cytokine/chemokine evaluation of NAG-MLP-injected nonhuman primates.

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