

Expressed Anti-HBV Primary MicroRNA Shuttles Inhibit Viral Replication Efficiently *In Vitro* and *In Vivo*

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The use of RNA interference (RNAi) to inhibit gene expression is potentially applicable in the treatment of viral infections such as hepatitis B virus (HBV) persistence. Although efficient HBV gene silencing by short hairpin RNA (shRNA) expressed from RNA polymerase (Pol) III promoters has been reported, constitutive high-level transcription may cause harmful side effects. Here, we report an approach that allows the use of a Pol II promoter to improve transcription regulation of expressed RNAi effectors. Pol II [cytomegalovirus (CMV)] or Pol III (U6) promoter cassettes that transcribe anti-HBV primary microRNA (pri-miR)-122 and pri-miR-31 shuttles were generated. In cultured cells both types of pri-miR-like sequences effected knockdown of markers of viral replication (>80%) and were processed to form intended 21-nucleotide guides. The concentration of CMV-expressed miRs was ~85-fold lower than the U6 shRNA-derived guide RNA. When cells were co-transfected with pri-miR expression cassettes, attenuation of independent RNAi-mediated gene silencing was not observed, which is in contrast to the action of U6 shRNA expression cassettes. The efficacy of the anti-HBV pri-miR shuttles *in vivo* was verified using the murine hydrodynamic injection model. Employing Pol II-expressed pri-miR mimics may be useful in the treatment of HBV infection, and potentially also for generic application in RNAi-based therapy.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection, which continues to be endemic to sub-Saharan Africa and parts of Asia, is often complicated by cirrhosis and hepatocellular carcinoma (reviewed in ref. 1). Licensed therapies are only partially effective, and developing improved treatment strategies to prevent the life-threatening sequelae of virus persistence remain an important medical priority.² Activation of the RNA interference (RNAi) pathway to effect specific HBV gene silencing^{3–8} has prompted enthusiasm for the potential of nucleic acid-based HBV treatment. RNAi

involves specific and powerful gene silencing through predictable complementary interaction between RNAi effectors and their targets.⁹ Naturally, RNAi plays an important role in regulating gene expression through the processing of endogenous miRs (reviewed in ref. 10), which control several cellular processes including organogenesis, apoptosis, cell proliferation, and tumorigenesis. miRs are transcribed by Pol II¹¹ as pri-miR hairpin-like structures, which are then processed to form precursor miRs (pre-miRs) within the nucleus. This step is catalyzed by Drosha (an RNase III enzyme) together with DGCR8, which is its double-stranded RNA-binding domain partner. After export from the nucleus, pre-miRs are processed by Dicer with associated double-stranded RNA-binding domain TAR RNA-binding protein. The resulting 19–24-base pair duplex is handed on to the RNA-induced silencing complex before selection of one strand as the mature miR guide. miRs are usually not entirely complementary to their targets, and bind to the 3' untranslated regions of cognate messenger RNA to induce translational suppression. When base pairing between guide and target is perfectly matched, the Ago2 component of RNA-induced silencing complex exerts silencing through site-specific cleavage of the guide complement.^{12,13}

The specific and powerful gene silencing that may be induced by RNAi has prompted investigation of RNAi-based therapeutic modalities to inhibit expression of pathology-causing genes, which include those of viruses such as HBV. Typically, exogenous RNAi-inducing sequences have been either synthetic short interfering RNA (siRNA) duplexes or expressed short hairpin RNA (shRNA) sequences.¹⁴ Synthetic siRNAs are similar to Dicer cleavage products and cause gene silencing by direct activation of RNA-induced silencing complex. shRNAs enter the RNAi pathway at an earlier stage and act as pre-miR mimics. Constitutively active Pol III promoters have been favored for transcribing shRNAs because of their ability to generate short, defined transcripts with a minimal requirement for regulatory elements within the transcript-encoding sequences. Several sites of the HBV genome have been targeted with synthetic and expressed RNA sequences, and impressive knockdown of markers of viral replication has been demonstrated.^{3–8} However, a recent finding that U6 Pol III-expressed anti-HBV shRNAs cause serious toxicity *in vivo* by saturating the endogenous miR pathway, is an important concern

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while considering the therapeutic application of expressed RNAi sequences.¹⁵ Tissue-specific and inducible Pol II promoters may therefore be preferable to Pol III regulatory elements, because they provide a better means of transcription control and dose regulation of expressed RNAi effectors. Some reports have demonstrated efficient silencing by Pol II RNAi expression cassettes, but this approach has been hampered by unpredictable and variable silencing efficacy of conventional hairpin sequences.¹⁶ Sequences upstream and downstream of the hairpins, which are incorporated into Pol II–derived transcripts, may interfere with processing of the silencers. In order to improve transcription control of potentially therapeutic sequences, we have taken advantage of the natural Pol II–mediated transcriptional control of cellular miRs. Anti-HBV sequences were incorporated into expression cassettes that encode mimics of pri-miR-31 (refs. 17,18) or pri-miR-122.¹⁹ Potent silencing of markers of viral replication was achieved *in vitro* and *in vivo* when anti-HBV pri-miR shuttle expression cassettes were placed under the control of Pol II or Pol III promoters.

RESULTS

Design of anti-HBV pri-miR–expressing plasmids

Pri-miR expression cassettes were designed by replacing guide and complementary sequences of natural miR-31 (refs. 17,18) and miR-122 (ref. 19) with those of an effective anti-HBV shRNA (U6 shRNA 5) described earlier.³ The wild-type sequences of the miRs were maintained as far as possible, and computer-aided prediction²⁰ of the secondary structure of the transcripts did not differ significantly from that of their respective wild-type miRs (Figure 1a). The final cassettes contained 51 nucleotides of wild-type sequence flanking either end of the pre-miR^{17,19} that was located downstream of a U6 promoter or within an exonic sequence of a cytomegalovirus (CMV) immediate early promoter enhancer expression cassette (Figure 1b). Unlike typical cellular miRs, the intended guide sequence was perfectly complementary to its HBV target, and was therefore expected to effect cleavage of all HBV transcripts.

miR-mediated inhibition of HBV surface antigen secretion from transfected cells

Initially, in order to assess efficacy against HBV *in vitro*, Huh7 cells were co-transfected with miR-31/5- and miR-122/5-expressing vectors together with the pCH-9/3091 HBV replication competent target plasmid²¹ (Figure 2a). Controls included a U6 shRNA-encoding plasmid (U6 shRNA 5), which we have previously shown to be effective against HBV,³ and also a vector in which the CMV promoter transcribed the shRNA 5 sequence. Compared with mock-treated cells, knockdown of 95–98% of viral antigen secretion was achieved by U6 shRNA 5-, U6 miR-31/5-, U6 miR-122/5-, CMV miR-31/5-, and CMV miR-122/5-expressing vectors (Figure 2b). CMV miR-122/5 was slightly less effective than the other miR vectors (85–90% knockdown). The vector encoding shRNA 5 derived from the CMV promoter caused the least efficient silencing (~60%).

miR-mediated inhibition of Firefly luciferase activity in transfected cells

The HBsAg secretion data were corroborated using a reporter gene plasmid (pCH Firefly Luc) to measure knockdown efficiency *in situ* (Figure 2c). In pCH Firefly Luc, the *preS2/S* sequence of pCH-9/3091 (ref. 21) was replaced with the *Firefly luciferase* open reading frame (ORF) with the targeted *HBx* ORF remaining intact (Figure 2a). Co-transfection of pCH Firefly Luc with miR-encoding vectors allows for the convenient quantitative measurement of anti-HBV efficacy *in situ* by determining luciferase reporter gene activity. Analysis showed that the Firefly luciferase activity was diminished significantly by U6 shRNA 5-, U6 miR-31/5-, U6 miR-122/5-, CMV miR-31/5-, and CMV miR-122/5-containing vectors. Each of these plasmids caused knockdown of ~75% as compared with controls (Figure 2c). Again, the CMV shRNA 5 vector did not inhibit Firefly luciferase activity significantly. Taken together, these data indicate that the incorporation of an anti-HBV sequence into miR-like structures of miR-31 or miR-122 enables expression of the silencing sequence from a Pol II or Pol III promoter without compromising silencing efficacy.

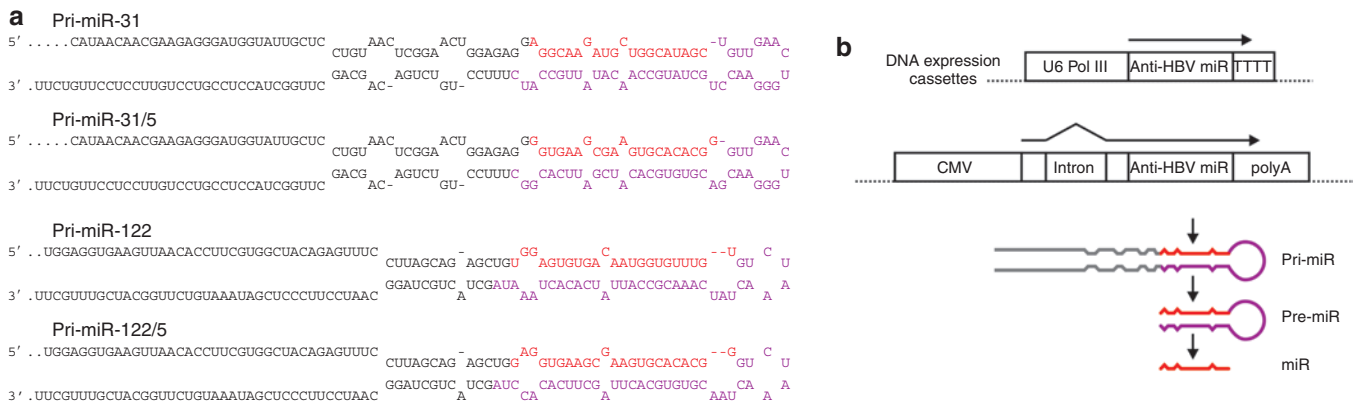


Figure 1 Pri-miR-31/5 and pri-miR-122/5 anti-HBV sequences. **(a)** Predicted structure and sequences of pri-miR-31 and pri-miR-122 (available at <http://microrna.sanger.ac.uk/>) with their anti-HBV derivatives, pri-miR-31/5 and pri-miR-122/5. The sequence of the putative pre-miRs generated after Drosha/DGCR8 processing is indicated in color (purple and red) and mature processed guide sequences are indicated in red only. **(b)** Schematic illustration (not to scale) of anti-HBV miR DNA expression cassettes showing putative processing of pri-miR and pre-miR to generate mature miR. The arrangement of the Pol II [cytomegalovirus (CMV)] or Pol III (U6) promoter, pri-miR-31/5- or pri-miR-122/5-encoding sequences, together with the transcription termination sequences are indicated. HBV, hepatitis B virus.

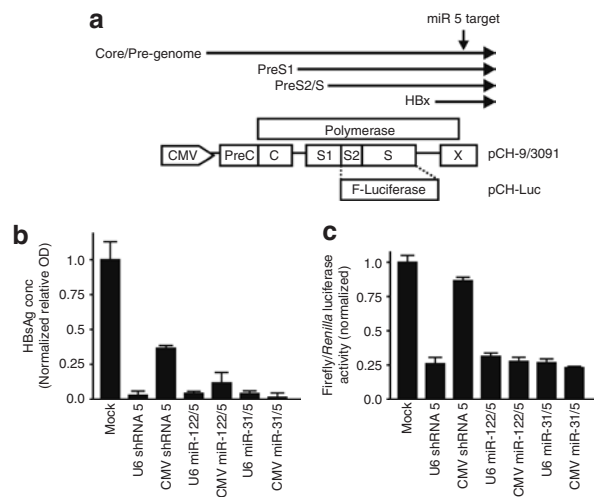


Figure 2 HBsAg secretion and reporter gene expression in Huh7 cells co-transfected with miR-encoding plasmids. **(a)** Organization of the hepatitis B virus (HBV) genome with open reading frames (ORFs) and sites within the pCH-9/3091 target vector. Four parallel arrows indicate the HBV transcripts which have common 3'-ends and include the miR-31/5, miR-122/5, and U6 short hairpin RNA 5 (shRNA 5) targets. The structure of the pCH-9/3091-derived pCH Firefly Luc target vector containing the *Firefly luciferase* ORF (F-luciferase) is indicated below. **(b)** HBsAg secretion from Huh7 cells co-transfected with plasmids encoding the indicated miR or shRNA cassettes, together with the HBV target plasmid. HBsAg measurements from quantitative enzyme-linked immunosorbent assay are given as a normalized mean relative to the corresponding measurements from mock-treated cells. The results are from three independent transfections, and the bars indicate the SEM. **(c)** Firefly luciferase reporter gene activity in transfected Huh7 cells. The measurements are given as a normalized ratio (\pm SEM) of Firefly activity to constitutively expressed *Renilla luciferase* activity, and were determined from three independent experiments. CMV, cytomegalovirus; OD, optical density.

Detection of processed miR-31/5 and miR-122/5 sequences

Northern blot hybridization analysis was carried out to detect processed products of the anti-HBV miR expression cassettes. RNA was extracted from transfected cells and **Figure 3** shows the signals obtained after hybridization to a probe that was complementary to the putative mature processed miR-31/5, miR-122/5, or shRNA 5 guides. The dominant processed product was detectable as a band of ~21 nucleotides in size, which is a length similar to that of naturally occurring mature miR-31 and miR-122 products.^{17,19} Interestingly, bands corresponding to RNA of 20 and 22 nucleotides in length were also detected in cells transfected with CMV miR-31/5 and U6 miR-31/5 (**Figure 3a**), which implies that processing of anti-HBV guide strands in the context of the miR-31 shuttle may be heterogeneous. Larger-molecular-weight miR/shRNA intermediates were detected in RNA extracted from cells transfected with U6 promoter-containing vectors but not from cells expressing the CMV miR-31/5 or CMV miR-122/5 cassettes. This suggests that complete processing of the CMV Pol II transcripts occurs more efficiently than that of the Pol III-expressed RNA. When compared with the U6 HBV shRNA 5-derived guide, which was included as a positive control of known high level expression, the mature miR-31 and miR-122 sequences were detected at up to 85-fold lower concentration. Interestingly, intracellular concentrations of miR-derived guides from U6 cassettes

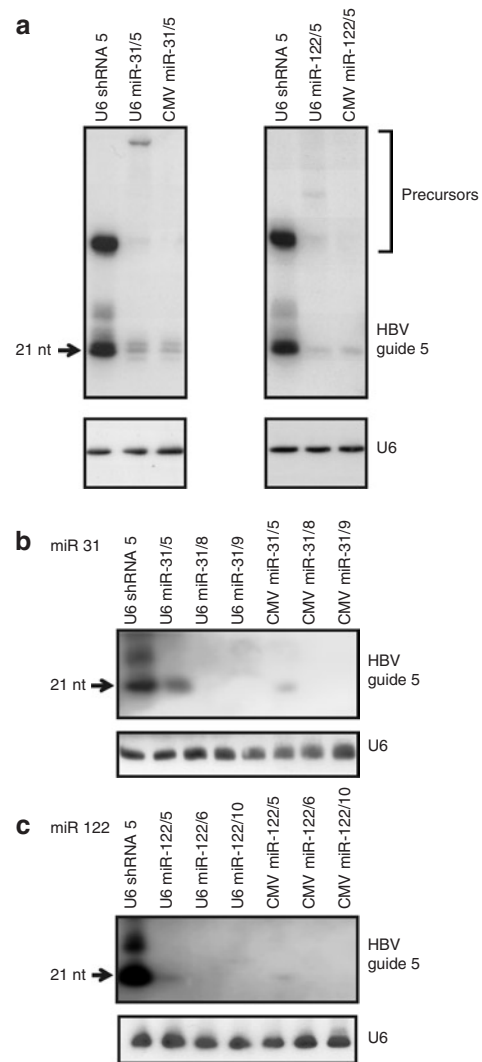


Figure 3 Northern blot hybridization analysis of expressed miR shuttle sequences that were extracted from HEK293 cells after transfection with plasmids encoding the indicated miR or short hairpin RNA (shRNA) cassettes. Hybridization was to a radiolabeled probe complementary to the putative mature anti-HBV guide 5 strand. Representative hybridization signals to detect **(a)** precursors of mature miRs and comparative concentrations of **(b)** mature miR-31/5 or **(c)** miR-122/5 sequences are shown. Blots were stripped and rehybridized to a probe complementary to endogenous U6 shRNA in order to confirm equal loading of cellular RNA (lower panels of **a**, **b**, and **c**). CMV, cytomegalovirus; HBV, hepatitis B virus.

were lower than for U6 shRNA 5. This could be the result of lower Pol III transcription efficiency of the longer miR-122/5 and miR-31/5 sequences. The detected guide strand signal was specific, as no bands were detectable when the probe was hybridized to RNA that had been extracted from cells transfected with similar pri-miR expression vectors that target different HBV sites (**Figure 3b** and **c**).

Assessment of off-target effects of miR-expression cassettes

The presence of duplex RNA within cells may lead to activation of a type 1 interferon (IFN) response, resultant programmed cell

death, and nonspecific gene silencing (reviewed in ref. 22). In order to assess activation of the IFN response, the ratios of cellular *IFN-β* to *GAPDH* messenger RNA concentrations were measured in transfected cells using a sensitive quantitative real-time PCR assay (Figure 4a). *IFN-β* messenger RNA was not significantly induced in any of the cell groups that had been transfected with miR-expressing vectors, while treatment with poly (I:C) (positive control) resulted in activation of *IFN-β* expression. *IFN-β* activation was not tested in Huh7 cells, because we (data not shown) and others²³ have observed that the IFN response is attenuated in this liver-derived line. In order to control for transfection efficiency, a plasmid expressing enhanced green fluorescent protein (eGFP) was also included in the assessment of IFN induction. No decrease in eGFP expression was observed in the presence of the miR-expressing vectors (data not shown), which further supports the idea that these shuttles are not toxic to cells.

The effect of miR-expressing vectors on independent RNAi-mediated gene silencing was also assessed. To determine this, a dual luciferase reporter plasmid (psiCHECK-8T) containing an independent HBV miR-31/8 target sequence downstream of the Renilla luciferase ORF was transfected together with pCMV miR-31/8 and each of the shRNA 5-, miR-31/5-, or miR-122/5-expressing vectors (Figure 4b). In accordance with previous observations that overexpression of shRNA from U6 Pol III promoter causes disruption of the endogenous miR pathway,¹⁵ the silencing of psiCHECK-8T target by pCMV miR-31/8 was diminished in the presence of pU6 HBV shRNA 5. This effect was, however, not observed when miR-122/5- or miR-31/5-expressing plasmids were co-transfected. These consequences are likely to be dependent on RNAi effector concentration, and this is in keeping with our finding that the intracellular pri-miR-derived guide sequences are present at lower concentrations than U6 shRNA 5 guides (Figure 3a). In order to corroborate this, knockdown was measured using decreasing concentrations of co-transfected pU6 HBV shRNA 5 with constant amounts of CMV miR-31/8 and psiCHECK-8T target (Figure 4c). Efficient miR-31/8-mediated knockdown was achieved at low concentrations of pU6 HBV shRNA 5. However, when the amount of pU6 HBV shRNA 5 was increased, the efficacy against HBV target 8 was diminished. Co-transfecting a similar range of pU6 HBV shRNA 5 concentrations with pCH-9/3091 HBV replication competent plasmid confirmed that potent silencing of HBsAg secretion is achieved by the RNAi effector (Figure 4d). These data further support the notion that disruption by pU6 HBV shRNA 5 of independent pCMV miR-31/8 silencing is influenced by the concentration of expressed shRNA 5.

Inhibition of markers of HBV replication *in vivo*

Concentrations of HBsAg were measured in the sera of mice that had been subjected to hydrodynamic tail-vein injection (HDI).²⁴ Each of the shRNA 5-, miR-31/5-, and miR-122/5-containing plasmids knocked down the serum viral antigen concentration by at least 95% (Figure 5a). This was observed when measurements were taken at both 3 and 5 days after HDI. Of the three plasmid vectors, pU6 shRNA 5 was the most efficient, and HBsAg was not detectable in the sera of mice injected with this plasmid. The number of circulating viral particle equivalents in the same mice was also measured using quantitative real-time

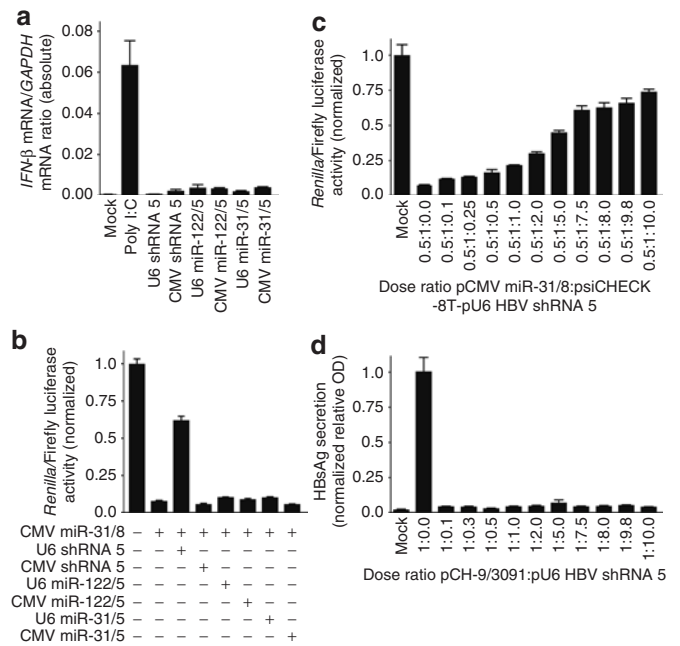


Figure 4 Assessment of off-target effects of miR-expression cassettes. **(a)** Interferon (IFN) response was assessed in HEK293 cells that were transfected with plasmids encoding the indicated miR-encoding cassettes or with poly (I:C). RNA was extracted from the cells 24 hours later and subjected to quantitative real-time PCR to determine concentrations of *IFN-β* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) messenger RNA (mRNA). The mean values (\pm SEM) of the normalized ratios of *IFN-β* to *GAPDH* mRNA concentrations are indicated from three independent experiments. **(b)** Attenuation of independent RNA interference-mediated silencing was assessed by co-transfection of Huh7 cells with plasmids expressing the indicated short hairpin RNA (shRNA) or miR cassettes, together with pCMV miR-31/8 and a psiCHECK-8T dual luciferase vector. The reporter plasmid contained the independent hepatitis B virus (HBV) miR-31/8 cognate sequence downstream of the Renilla luciferase open reading frame. Measurement of *Renilla:Firefly* luciferase activity was used for assessing the effects of shRNA 5-, miR-31/5-, or miR-122/5-expressing plasmids on miR-31/8 silencing of its target. **(c)** Effect of the amounts of pU6 HBV shRNA 5 used for transfection on attenuation of cytomegalovirus (CMV) miR-31/8 silencing. The indicated mass ratios of pCMV miR-31/8 to psiCHECK-8T to pU6 HBV shRNA 5 vectors were co-transfected. Again, measurements of *Renilla:Firefly* luciferase activities (\pm SEM) were used for assessing the effects of decreasing amounts of pU6 HBV shRNA 5 on miR-31/8 silencing of its target. **(d)** Inhibition of HBsAg secretion from Huh7 cells that were transfected with decreasing amounts of pU6 HBV shRNA 5. The indicated mass ratios of pU6 HBV shRNA 5 to HBV replication competent pCH-9/3091 vectors were used for transfection and the HBsAg concentrations in the culture supernatants were determined 48 hours thereafter. Normalized mean relative optical density (OD) readings (\pm SEM) from enzyme-linked immunosorbent assays are represented.

PCR at days 3 and 5 (Figure 5b). The results corroborate the HBsAg determinations, in that pU6 shRNA 5, pCMV miR-31/5, and pCMV miR-122/5, each decreased the number of circulating viral particle equivalents by at least 95%. HBV DNA replication intermediates were also measured in the liver tissues of representative animals that had been subjected to HDI experimentation (Figure 5c). HBV duplex linear and relaxed circular DNA were detectable only in the mock-treated animals, but not in any of the mice that had been co-injected with CMV miR-31/5, CMV miR-122/5, or U6 shRNA 5 plasmids. The size of the HBV DNA bands from mock-treated mice did not correspond to any of the

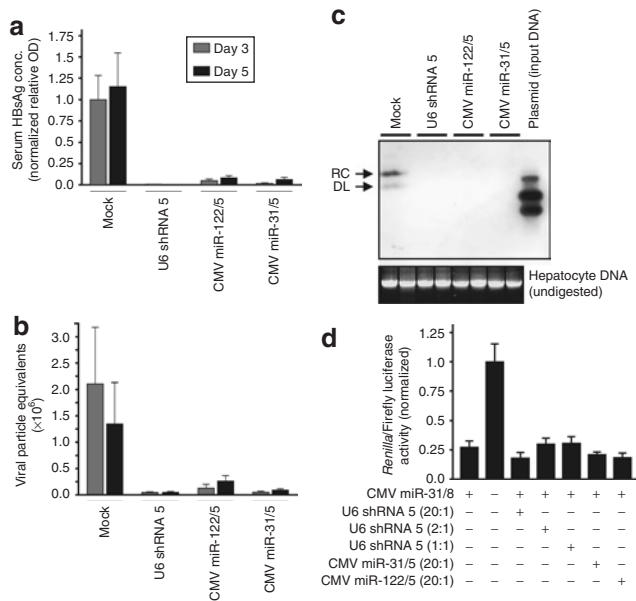


Figure 5 Effects of miR sequences on markers of hepatitis B virus (HBV) replication *in vivo* using the hydrodynamic tail-vein injection (HDI) model of HBV replication. **(a)** Serum HBsAg concentrations and **(b)** circulating viral particle equivalents were determined at days 3 and 5 after HDI of mice with pCH-9/3091 HBV target and plasmids encoding cytomegalovirus (CMV) miR-31/5, CMV miR-122/5, or U6 short hairpin RNA 5 (shRNA 5) sequences. The results are expressed as mean values (\pm SEM) from at least four mice. **(c)** Southern blot analysis of HBV DNA replication intermediates extracted from two representative animals from each of the groups of mice that had been subjected to the HDI procedure (upper panel). HBV duplex linear (DL) and relaxed circular (RC) replication intermediates were detectable only in the mock-treated animals. pCH-9/3091 was loaded as a control to verify that HBV replication intermediates did not correspond to input DNA. The lower panel is a representation of the separated extracted DNA after ethidium bromide staining and before Southern transfer and hybridization. **(d)** In order to assess the effects of miR shuttles on independent silencing *in vivo*, mice were subjected to HDI with the psiCHECK-8T vector, together with the indicated RNA interference expression cassettes. Where relevant, the ratios of the CMV miR-31/5, CMV miR-122/5, and U6 shRNA 5 to CMV miR-31/8 vectors are indicated in parentheses. The normalized mean values of *Renilla*:*Firefly* luciferase activities (\pm SEM) were determined in liver homogenates 3 days after plasmid injection. mRNA, messenger RNA; OD, optical density.

pCH-9/3091 bands, thereby signifying that the detected double-stranded and relaxed circular HBV DNA were not the same as input plasmid DNA.

For the purpose of assessing possible disruption of independent RNAi-mediated silencing and toxicity *in vivo* caused to hepatocytes by pri-miR shuttles, mice were also injected with the psiCHECK-8T dual luciferase reporter plasmid and various anti-HBV expression cassettes (Figure 5d). Firefly and *Renilla* luciferase activities in liver homogenates were measured 3 days after HDI. Selective and efficient silencing of *Renilla* luciferase activity was achieved with pCMV miR-31/8. This knockdown was not attenuated by co-injection of 20-fold excess amount of U6 shRNA 5, CMV miR-122/5, or CMV miR-31/5. This indicates that, under the experimental conditions described here, independent silencing was unaffected by U6 shRNA or miR shuttle expression. When using the HDI model, direct assessment of hepatotoxicity caused by miR mimics is complicated by the damage to liver cells caused by the

release of enzyme markers that is inherent to the injection procedure itself. As a surrogate indicator to assess damage to liver cells caused by pri-miR shuttles, untargeted and constitutively active psiCHECK-8T-derived Firefly luciferase activity was independently evaluated in the groups of mice. When compared with the animals receiving no RNAi effector, the mice that had received the miR shuttles showed undiminished Firefly luciferase activity in liver homogenates (data not shown). Collectively, these data show that miR mimics generated from CMV miR-31/5 and CMV miR-122/5 are specific silencers of HBV replication *in vivo* with negligible effects on independent RNAi-mediated silencing. Moreover, the efficacy of the miR-expression cassettes is comparable with those of the U6 shRNA 5 sequences.

DISCUSSION

Although recent studies have demonstrated efficient silencing of HBV replication by activating the RNAi pathway,³⁻⁸ some hurdles remain before the goal of therapeutic application of this approach is realized. Particularly important is limiting of unintended effects, which include off-target silencing of cellular RNA, disruption of the endogenous miR pathway and immunostimulation. Both synthetic and expressed sequences are being widely used to achieve RNAi-mediated HBV gene knockdown.¹⁴ Unlike with DNA expression cassettes, dose regulation and delivery is easier to achieve with synthetic siRNAs. Nonetheless, expressed RNAi sequences have significant advantages, which include better stability of DNA templates, ability to achieve sustained silencing by continuously transcribed RNAi effectors, as well as compatibility of expression cassettes with incorporation into both recombinant viral and nonviral vectors. These properties render expression cassettes well suited for the treatment of chronic diseases such as persistent HBV infection. Constitutively active Pol III promoters have traditionally been used for expressing RNAi effectors. However, these regulatory elements have been reported to have serious toxic effects *in vivo*, which result from shRNA overexpression and saturation of the endogenous miR pathway.¹⁵ Against this background, the use of Pol II promoters to express RNAi effectors offers the advantage of being able to refine transcription control and thereby limit unwanted effects. This study shows that potent knockdown of markers of HBV replication is attained *in vitro* and *in vivo* when an antiviral guide is incorporated into exonic pri-miR mimics that are transcribed from a Pol II promoter. miR-122 and miR-31 backbones were selected because these sequences were predicted to favor intrahepatic processing of adapted anti-HBV shuttles. miR-122 is liver-specific¹⁹ and pri-miR-31 is efficiently processed by Drosha.¹⁷ The demonstration that the anti-HBV miRs do generate an intended guide strand of ~21 nucleotides confirms that these sequences function as pri-miR mimics and are processed in a manner similar to those of natural pri-miRs. Potent silencing of markers of HBV replication was observed, with no evidence of toxicity or disruption of independent miR-mediated silencing. Also, data from our investigations have shown that anti-HBV pri-miR sequences that target different sites within the virus are capable of efficient silencing (Figures 4b and 5d). These findings suggest that the design of Pol II pri-miR shuttles described here is potentially valuable for treating HBV infection and may be useful for generic application in RNAi-based therapy.

Significant progress has been made in understanding the mechanisms of pri-miR processing and we aimed to utilize these recent insights to ensure optimal design of anti-HBV RNAi expression cassettes. Zeng *et al.*^{17,25} have shown that Drosha substrate preference is for RNA hairpins that bear long terminal loops, and that pre-miRs are generated by cleavage that occurs two helical turns from the junction of the pri-miR loop and stem sequences. By contrast, Han *et al.*²⁶ reported that the terminal loop sequence is not essential. According to this alternative mechanism, the initial binding of DGCR8 to pri-miRs requires single-stranded hairpin-flanking regions. Thereafter, Drosha is recruited to cleave the pri-miR ~11 nucleotides from the stem-single-stranded RNA junction. The reason for the apparent differences between these proposed pri-miR processing mechanisms is unclear, but it is possible that both operate naturally. The anti-HBV pri-miR shuttles that we used here accommodate Drosha/DGCR8 processing by either of the mechanisms. Although other studies have reported silencing by pre-miR or shRNAs expressed from Pol II promoter elements,^{27–36} these cassettes are of variable and sometimes poor silencing efficiency, and therefore this approach has not gained widespread acceptance. Importantly, if flanking single-stranded RNA sequences are of importance in the nuclear processing of pri-miR,²⁶ then it is likely that pre-miR and shRNA expressions from Pol II promoters will not be as efficient as those from the pri-miR mimics described here.

Recently, in an attempt to improve efficacy and overcome problems of viral escape mutation, long hairpin RNA-expressing sequences have been used for silencing HBV.³⁷ These cassettes were intended to be capable of generating different siRNAs to target independent HBV sites simultaneously. However, the use of long hairpin RNA expression cassettes seems to be limited by intracellular Dicer processivity and difficulties associated with controlling guide strand bias of multiple siRNAs. In addition to their production from Pol II promoters, another interesting property of miRs is that they are often naturally derived from polycistronic precursor sequences.¹⁰ This is a characteristic that could be adapted for the formation of multimeric therapeutic silencing sequences,³⁶ which would be useful for overcoming viral escape. Current investigations in our laboratory are aimed at assessing the use of liver-specific Pol II promoters and production of multimeric pri-miR shuttles that target different HBV sequences.

MATERIALS AND METHODS

Anti-HBV miR sequences. Initially, DNA encoding pre-miR-31 and pre-miR-122 sequences with a guide targeting HBV were generated by PCR-based primer extension of partially complementary pre-miR-31/5 and pre-miR-122/5 forward and reverse primers. The anti-HBV guide of miR-31/5 targets HBV coordinates 1,575–1,595, and miR-122/5 targets HBV coordinates 1,575–1,597.

The oligonucleotide sequences were pre-miR-31/5 forward: 5'-GTAACTCGGAAGCTGGAGAGGGGTGAAGCGAAGTGCACACGGGTTGAACTGGGAACGACG-3', pre-miR-31/5 reverse: 5'-CTGCTGTCAGACAGGAAAGCCGTGAATCGATGTGCACACGTCGTTCCAGTTCAACCCTG-3', pre-miR-122/5 forward: 5'-GAGTTTCCTTAGCAGAGCTGGAGGTGAAGCGAAGTGCACACGGGTCTAACTAACGTGTGCA-3' and pre-miR-122/5 reverse: 5'-GGATTGCTTACAGTAGCTAGCTAGGTGTGAAGCTAAGTGCACACGTTAGTTTAGACCCGTGCA-3'. The pre-miR-31/5 and pre-miR-122/5 products were purified and used as the template for a second round

of PCR amplification with forward and reverse pri-miR primers, respectively. The primer sequences were pri-miR-31 forward: 5'-GCTAGCCA TAACAACGAAGAGGGATGGTATTGCTCTGTAACTCGGAACTG GAGAGG-3', pri-miR-31 reverse: 5'-AAAAAACTAGTAAGACAAG GAGGAACAGGACGGAGGTAGCCAAGCTGCTGTGTCAGACAG GAAGC-3'; pri-miR-122 forward: 5'-GACTGCTAGCTGGAGGTGAAG TTAACACCTTCGTGGCTACAGAGTTTCTTAGCAGAGCTG-3' and pri-miR-122 reverse: 5'-GATCACTAGTAAAAAGCAAACGATGCCA AGACATTTATCGAGGGAAGGATTGCCTAGCAGTAGCTA-3'. The U6 promoter was also amplified using standard PCR conditions from a U6 promoter-containing plasmid.³⁸ The primer sequences were U6 forward: 5'-GATCAGATCTGGTTCGGCAGGAAGAGGGCC-3' and U6 reverse: 5'-GCTAGCGGTGTTTCGTCTTTCCACA-3'. DNA fragments encoding pri-miR-31/5, pri-miR-122/5, or the U6 promoter were each ligated into pTZ57R/T (InsT/Aclone PCR Cloning Kit; Fermentas, Hanover, MD) to generate pTZ pri-miR-31/5, pTZ pri-miR-122/5, and pTZ-U6 respectively. For generating Pol III-driven pri-miR vectors, pri-miR-31/5 and pri-miR-122/5 fragments were cloned downstream of the U6 promoter in pTZ-U6. Pri-miR-31/5 was excised from pTZ pri-miR-31/5 by digesting with NheI and ScaI, and pri-miR-122/5 was removed from pTZ pri-miR-122/5 after NheI and EcoRI restriction. These fragments were inserted into SpeI and ScaI, or SpeI and EcoRI sites of pTZ-U6 to produce pU6 pri-miR-31/5 and pU6 pri-miR-122/5, respectively. The method of generation of the pU6 shRNA 5 vector has been described earlier.³ Pol II-driven pri-miR vectors were generated by inserting pri-miR-31/5, pri-miR-122/5, and shRNA 5 sequences downstream of the CMV of pCI-neo (Promega, Madison, WI). pri-miR-31/5 was excised from pTZ pri-miR-31/5 with Sall and NheI and ligated to complementary overhangs of pCI-neo (which had been digested with XhoI and XbaI) to form pCMV pri-miR-31/5. pCMV pri-miR-122/5 was generated by inserting a NheI and XbaI restriction fragment from pTZ pri-miR-122/5 into a pCI-neo backbone that had been digested with the same restriction enzymes. pTZ pri-miR-31/8 (HBV target 1,678–1,700) and pCMV pri-miR-31/8 (HBV target 1,678–1,700) expression cassettes targeting different sites within the HBx ORF were generated using similar procedures.

Target plasmids. pCH-9/3091 has been described earlier.²¹ The pCH Firefly Luc vector was prepared by replacing the *preS2/S* ORF of pCH-9/3091 with Firefly luciferase-encoding DNA. A *Firefly luciferase* sequence was amplified from pGL4 (Promega, Madison, WI) using PCR. The forward primer comprised sequences complementary to HBV sequences from coordinates 129–159 (including a naturally occurring *XhoI* restriction site) and 5' *Firefly luciferase* sequences. In this primer, the position of the *Firefly luciferase* initiation codon is equivalent to that of the translation initiation codon of the middle HBs protein. The reverse primer included sequences complementary to the 3'-end of the *Firefly luciferase* ORF, as well as a *SpeI* restriction site. The PCR primer sequences were Firefly Luc forward: 5'-ACTGCTCGAGG ATTGGGGACCCTGCGCTGAACATGGAAGACGCCAAAAAC-3' and Firefly Luc reverse: 5'-ACTGACTAGTTTACACGGCGATCTTTCC-3'. The PCR product was cloned into pTZ57R/T to generate pTZ Firefly Luc. The *Firefly luciferase* sequence was then excised from pTZ Firefly Luc with *XhoI* and *SpeI* and inserted into the *XhoI* and *SpeI* sites of pCH-9/3091 to generate pCH Firefly Luc. For generating psiCHECK-8T, containing the miR 8 target, primer 8T forward 5'-CAATGTCAACGACCACCT-3' and primer 8T reverse 5'-ACTAGTGCCTCAAGGTGCGGT-3' were used for amplifying nucleotides 1,678–1,702 of the HBV genome and for introducing a *SpeI* site at the 3'-end of the amplicon. The purified fragment was ligated into the pTZ57R/T PCR cloning vector, and the insert was removed with *Sall* and *SpeI* and then ligated into the *XhoI* and *SpeI* sites of the psiCHECK 2.2 derivative of psiCHECK 2 (Promega, WI). The resulting plasmid, psiCHECK-8T, contained the HBV target site downstream of the Renilla luciferase ORF. The psiCHECK 2.2 plasmid, a gift from Dr. Marc Weinberg, contained additional *SacI*, *EcoRI*, *SmaI*, *SpeI*, and *Sall* sites between the *NotI* and *XhoI* sites of psiCHECK 2. All the plasmid sequences

were verified in accordance with standard dideoxy chain termination protocols (Inqaba Biotechnology, Pretoria, South Africa).

Cell culture. Culture and transfection of Huh7 and HEK293 lines were carried out as described earlier.³ For determining the effects of miR-31/5- and miR-122/5-encoding plasmids, cells were transfected with a combination of 80 ng of pCH-9/3091 (ref. 21) or pCH Firefly Luc target vector and 800 ng of shRNA 5, miR-31/5- and miR-122/5-derived plasmids, or vector lacking the miR cassettes. For assessing the effects of miR-31/5- and miR-122/5-expressing plasmids on independent RNAi-mediated silencing, cells were seeded into 24-well dishes at a density of 35–40% and transfected with 80 ng of psiCHECK-8T, 40 ng of pCMV miR-31/8, and 780 ng of shRNA 5 or miR 5 expression plasmids. In the case of transfections with the pCH Firefly Luc target vector, a plasmid that constitutively produces *Renilla* luciferase under control of the CMV promoter (phRL-CMV; Promega, Madison, WI) was included in order to control for transfection efficiency. The effects of the plasmid dose of pU6 shRNA 5 on independent silencing by pCMV miR-31/8 were determined by transfecting with pU6 shRNA 5 in amounts ranging from 0 to 800 ng. Similarly, for determining the silencing potency of pU6 shRNA 5 against pCH-9/3091, pU6 shRNA 5 was used for transfection in amounts ranging from 0 to 800 ng. A constant amount of 80 ng of psiCHECK-8T and pCH-9/3091 were used for transfection in each well. Backbone plasmid was included in each case so as to ensure that equal amounts of total plasmid DNA were used for transfection. The measurement of IFN response, HBsAg, and the activities of *Renilla* and Firefly luciferase were determined as described earlier.³⁷ A plasmid vector that constitutively produces eGFP³⁹ was also included in each co-transfection so as to verify equivalent transfection efficiencies using fluorescence microscopy. Northern blot analysis of total RNA extracted 3 days after transfection was carried out as described earlier.³⁷ The miR-31/5, miR-122/5, and shRNA 5 guide probe oligonucleotide was 5'-GACTCCCGTCTGTGCCCTTCTCA-3'.

Testing of anti-HBV efficacy of miR sequences in vivo using the HDI model of HBV replication. The murine HDI method^{24,37} was employed for determining the *in vivo* effects of miR-expressing vectors on the markers of HBV replication and reporter gene expression. All experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. For the purpose of assessing the effects on viral replication, the injected solutions included a combination of three plasmid vectors: 5 µg target DNA (pCH-9/3091); 5 µg anti-HBV sequence (pU6 shRNA 5, pCMV miR-31/5, or pCMV miR-122/5 plasmid) or mock (pTZ backbone); and 5 µg pCI-neo eGFP (a control for hepatic DNA delivery, constitutively expressing the eGFP marker gene³⁹). Serum HBsAg concentration and circulating viral particle equivalents were measured as described.³ The mice were killed at day 5 after HDI, and the livers were removed. Total DNA was extracted⁴⁰ and subjected to agarose gel electrophoresis without restriction digestion before being processed for Southern blot analysis using Rapid-hyb solution (Amersham, Piscataway, NJ). pCH-9/3091 (ref. 21) was run alongside as a control for input DNA. For generating a probe, HBx DNA was amplified with HBx forward (5'-GATCAAGCTTTCGCCAACTTACAAGGCCTT-3') and HBx reverse (5'-GATCTCTAGAACAGTAGCTCCAAATTCTTTA-3') primers. The PCR products were purified and used as the template for random-primed labeling with the HexaLabel DNA Labeling kit (Fermentas, WI), in accordance with the manufacturer's instructions. In order to determine the effects of miR on reporter gene activity *in vivo*, mice were administered 0.5 µg reporter target DNA (psiCHECK-8T), 5 µg pCH-9/3091, combinations of anti-HBV plasmids (pCMV miR-31/8, pU6 shRNA 5, pCMV miR-31/5, or pCMV miR-122/5), or mock (pCI-neo backbone). The mice were killed 3 days after HDI, and their livers were removed and homogenized in phosphate-buffered saline; thereafter, the activities of *Renilla* and Firefly luciferase were determined as described earlier.³⁷

Statistical analysis. Analysis of statistically significant differences was carried out using the Student's paired two-tailed *t*-test.

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