Expression of shRNA From a Tissue-specific pol II Promoter Is an Effective and Safe RNAi Therapeutic

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It has been observed that overexpression of some short-hairpin RNAs (shRNAs) can induce acute cytotoxicity. This has raised concerns about the safety of using RNA interference (RNAi) technology as a potential therapeutic tool. We have sought to address this challenge of expression control by developing a mono-cistronic vector for the tissue-specific expression of an shRNA from a liver-derived polymerase (pol) II promoter. This new construct efficiently induces target silencing in hepatoma cells *in vitro* and in mouse livers *in vivo*. In order to demonstrate the therapeutic potential and improved safety of this approach, we selected an shRNA targeting the envelope surface antigen (sAg) of hepatitis B virus (HBV), which is among the most toxic when expressed from the commonly used U6 promoter. Packaging it as a double-stranded DNA into an adeno-associated virus (AAV) pseudotype 8 and delivering it at a high particle dose (1×10^{12}) to HBV transgenic mice resulted in the stable reduction of serum sAg to 85% of starting levels, without any concomitant sign of liver damage. With this improved tolerability, the liver-specific pol II shRNA expression persisted for more than one year after the injection. We conclude that this pol II shRNA expression system combined with a potent delivery vector represents an effective alternative to either U6-based strategies or systems that achieve tissue specificity through the use of additional elements.

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INTRODUCTION

Vector-mediated delivery of short-hairpin RNA (shRNA) for inducing stable, target-specific silencing by RNA interference (RNAi) holds great therapeutic potential in viral infections and aberrant gene disorders. Polymerase (pol) III promoters such as H1 and U6 remain the standard for use in driving shRNA expression. The relatively short lengths of these promoters (a few hundred base pairs) make them easy to clone and package into vectors with limited carrying capacity. Moreover, the transcription initiation and termination sites are well defined, facilitating accurate shRNA design. Although these advantages of pol III promoters make them valuable research tools, some features of these sequences render them less advantageous for certain therapeutic applications.

The first of these disadvantages is the overly robust and constitutive activity of pol III promoters across multiple cell types. On their own, pol III promoters do not provide the spatial or temporal control that is desirable in treating some human diseases. In order to address this issue, some groups of researchers have employed additional regulatory elements to augment the standard pol III system. Such approaches include Tet-regulation,[1] Cre-dependent activation,[2] and many others.[3] The therapeutic application of such systems can be challenging for two reasons. First, the additional elements must be delivered to and actively coexpressed in patients, and this can be particularly problematic if the additional element is a foreign protein. Second, the inclusion of these sequences could enlarge the shRNA expression cassette beyond the packaging capacity of small yet efficient viral vectors such as adeno-associated virus (AAV).

Another problem with using standard pol III promoters with high levels of expression is the saturable nature of the endogenous microRNA (miRNA) processing factors, Exportin-5 and RISC.[4,5] In some cases, such saturation appears to cause cytotoxicity and tissue damage.[6] The use of weaker pol III promoters in place of U6, a particularly strong member of the promoter class, may well address this saturation issue, but not the cell-type promiscuity. In addition to lacking desirable spatial or temporal control, pol III alternatives are more limited in number than the existing plethora of well-characterized pol II promoters that are available to choose from, some of which have been tested clinically and have been proven to be tissue-specific. In one such example, a promoter that is not transcriptionally active to any appreciable extent in nonliver tissue (liver specific) is used for expressing human clotting factor IX in human patients with hemophilia B.[7] Although it would be advantageous to likewise restrict shRNA transcription, the relatively poorly understood requirements of flanking sequences for efficient transcription and processing pose a challenge.[8]

As an alternative to optimizing direct expression of shRNAs, many have turned to embedding shRNA sequences within a miRNA context,[9,10] and some consider this process the next generation of RNAi technology.[11] Endogenous miRNAs are often transcribed from pol II promoters, and are therefore already

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adapted for this use. Such artificial miRNAs have been successfully employed to control cell proltiferation[12] and metastasis,[13] and to create shRNA expression libraries.[14] However, this strategy requires the additional step of excision from the longer primary miRNA sequence by the Drosha-DGCR8 complex.[15] While accommodating the use of pol II promoters and assuring generation of a mature sh/miRNA, this additional step may add another point of competition with endogenous miRNAs, thereby increasing the processing complexity as well as the risk of processing saturation.

We reasoned that an optimized shRNA expression system that could address all of these concerns would employ a tissue-specific and/or regulatable pol II promoter, have a moderate level of activity, and be engineered to drive transcription of a minimal hairpin. In order to achieve this, we evaluated several constructs, modulating promoters, hairpin overhangs, and termination signals to identify the most efficient design. We demonstrate feasible application as a therapeutic by delivering the optimized shRNA expression cassette within a potent viral vector (double-stranded AAV pseudotype 8 (dsAAV8)) to a clinically relevant mouse model of hepatitis B viral (HBV) infection.[16] In addition to achieving stable and potent *in vivo* silencing of *HBV* gene expression, the use of a moderately active and liver-specific promoter also abrogated the toxicity previously observed in association with U6-driven expression of the same hairpin sequence.

RESULTS

Optimization of pol II shRNA expression cassette design

An earlier study by Xia *et al.* suggested that optimization of the distances from the promoter to the hairpin and from the hairpin to the termination site is critically important for the successful induction of RNAi.[17] On the basis of this general design, our first constructs consisted of a pol II promoter followed by a fully complementary 25-mer hairpin against firefly luciferase with a 5 nt loop, and ended with the minimal polyadenylation (polyA) signal (used by Xia *et al.*), with 6 nt cloning sites between each of these elements (**Figure 1a**). In order to restrict therapeutic RNAi to the liver, one of the promoters tested was the liver-specific ApoE/hAAT (Ah) fusion promoter consisting of the hepatic control region from the apolipoprotein E gene promoter and the promoter region from the human α-1-antitrypsin (*hAAT*) gene.[18] This promoter was used in a clinical trial after it was shown in preclinical studies to be transcriptionally silent in nonhepatic tissues (ref. 7 and M.A.K., L.C. Linda Couto, G.P. Glenn Pierce, K.H. Katherine High, unpublished results). As the activity of this promoter was found to be significantly weaker *in vitro* than *in vivo*,[18] for initial screening purposes we also evaluated the human ubiquitin C (UbiC) and cytomegalovirus immediate-early enhancer/chicken-β-actin hybrid promoters *in vitro*. We first tested all constructs for their silencing activity, using dual-luciferase assay (Promega, Madison, WI). Of the two ubiquitously active promoters, UbiC proved the more efficient, inducing 71% silencing relative to an empty control

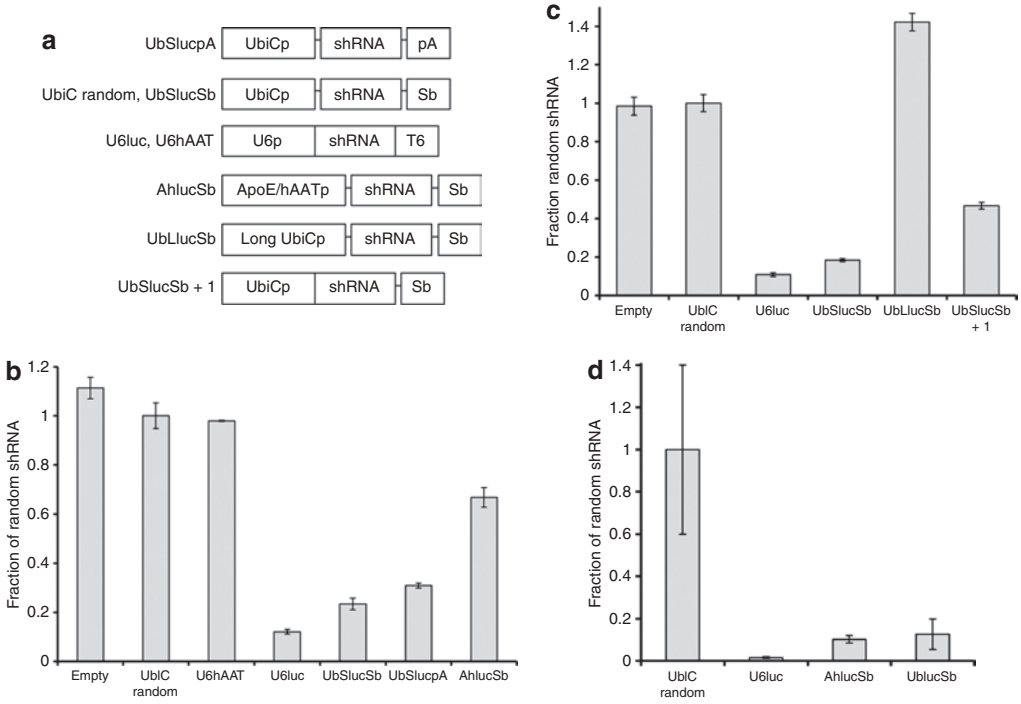

Figure 1 Luciferase silencing by pol II promoter-driven shRNA *in vitro* and *in vivo*. (**a**) Illustration of constructs evaluated in **b**, **c**, and **d**. UbiCp, minimal human ubiquitin promoter; long UbiCp, UbiCp with additional 877 bp of exon and intron sequence; ApoE/hAAT, liver-specific pol II fusion promoter; pA, minimal polyA; Sb, 14 nt U1 3′-box. shRNA sequence targets firefly luciferase (Fluc), human α-1-antitrypsin (hAAT), or random sequence. Gaps between boxes represent cloning sites. The absence of a gap indicates that there is no intervening sequence (*e.g.*, shRNA at +1). Not drawn to scale. (**b**) Measurements of relative Fluc activity levels in mouse Hep1A cells, normalized to co-transfected renilla luciferase and expressed as a fraction of the reading in cells that received a random-sequence shRNA construct. Labels are according to hairpin construct: Empty, pBSKS II+. (**c**) Comparison of Fluc silencing by various UbiC promoter constructs. (**d**) Measurements of Fluc silencing in live mice, normalized to hydrodynamically co-transfected RSV-hAAT and expressed as a fraction of the reading in mice that received a random-sequence shRNA. All error bars represent ±1 SD.

as compared to 41% induced by the cytomegalovirus hybrid promoter (data not shown). Therefore we used the UbiC promoter in testing subsequent construct variations.

Because overhanging single-stranded sequences were found to inhibit RNAi induction by short double-stranded RNA,[17,19] we first sought to optimize the predicted overhangs of our pol II promoter-driven shRNAs. We began with the 3′-overhang (sequence between shRNA and termination) by focusing on the termination signal. Similar in effect (though different in mechanism) to a hepatitis delta virus ribozyme used previously,[20] the 14 nt 3′-box termination sequence from the U1 snRNA directs cleavage immediately upstream of the signal, which would be ideal if it were closely juxtaposed at the 3′-end of an shRNA expression sequence. While cleavage is reportedly dependent on coupling with the U1 promoter,[21,22] it was also found to take place *in vitro* and in the absence of active transcription.[23] Encouraged by this finding, we replaced the 58 nt minimal polyA sequence with this 14 nt sequence, with the aim of minimizing the construct size and the extent of 3′ shRNA overhang (UbSlucpA and UbSlucSb in **Figure 1a**). The improvement in silencing efficacy over the minimal polyA-terminated constructs was an average of 7% (**Figure 1b**). While we cannot claim this improvement is the result of a shorter 3′-overhang, the use of this termination signal is at least as good as, if not better than, the minimal polyA. For this reason, in combination with the ease of working with a shorter signal and the theoretically shorter 3′-overhang, we used the 3′-box termination signal (designated Sb in construct names) for subsequent shRNA expression cassettes.

The second pol II–driven shRNA parameter that we optimized was the 5′-overhang length (sequence between promoter and shRNA). The source vector of the UbiC promoter, pUB6/V5-His B (Invitrogen, Carlsbad, CA), designates the promoter region as including exonic and intronic sequences to enhance translation. Placing this entire region upstream of the hairpin effectively adds an 877 nt leader in addition to the 6 nt NdeI site as a 5′-overhang on the shRNA (UbLlucSb, **Figure 1a**). As predicted by the observations of Xia *et al.*, silencing activity was completely abrogated relative to the result from an otherwise identical construct employing only the promoter sequence (UbSlucSb, **Figure 1c**). In addition to testing the effect of a long 5′-extension to the shRNA, we also evaluated the effect of placing the hairpin at +1 from the promoter (UbSlucSb+1, **Figure 1a**). This resulted in ~28% less relative silencing activity as compared to a construct with the hairpin at +7 following an NdeI site (UbSlucSb+1 and UbSlucSb, **Figure 1c**). The liver-specific Ah promoter construct (AhlucSb) induced only 33% silencing relative to the random-sequence shRNA but, as mentioned earlier, the activity of this promoter *in vitro* is limited. None of the pol II promoters performed at the level of the U6-driven shRNA (82% silencing, **Figure 1b**). This is likely to be the combined result of a longer trigger (29-mer stem) and a higher level of expression.

Before adapting the expression cassette for therapeutic evaluation, we evaluated the *in vivo* silencing activity of the 3′-box terminated construct with either the liver-specific ApoE/hAAT (AhlucSb) or the ubiquitous UbiC (UbSlucSb) promoter. BALB/c mice were hydrodynamically co-transfected with the respective shRNA expression plasmid together with plasmids for the expression of firefly luciferase (Fluc, the target) and hAAT (to normalize for transfection efficiency). At 3 days after transfection, co-delivery of either AhlucSb or UbSlucSb resulted in an ~90% reduction in normalized luciferase activity relative to a random hairpin control (**Figure 1d**). Silencing by the U6 construct was higher, at 98.5%, and this exemplifies the trade-off between activity and control inherent to the decision to select either a pol III or a pol II promoter.

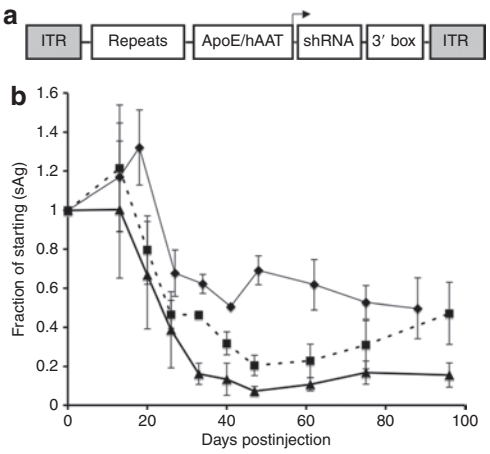

Figure 2 Pol II promoter-driven shRNA as HBV therapeutic in mice. (**a**) Schematic of shRNA expression construct packaged as a double-strand in pseudotyped AAV8. Labels: repeats (human alphoid repeat stuffer sequence), ApoE/hAAT (liver-specific promoter), shRNA (hairpin sequence), 3′-box (termination signal), ITR (inverted terminal repeats for packaging). (**b**) Serum sAg levels in transgenic HBV mice over a 96-day period. All constructs are as illustrated in **a**, with shRNAs and doses as follows: 25-mer shRNA against luciferase as negative control, 3×10^{11} (diamonds, $n = 3$), and two groups of 25-mer shRNA against sAg, 3×10^{11} (squares, $n = 3$, $P < 0.034$) and 1×10^{12} (triangles, $n = 4$, $P < 0.006$). Mean values of serum sAg are expressed as fractions of pre-injection levels. Error bars represent ±1 SD. *P* values were calculated for day 75 after the injection, with respect to negative controls.

Pol II–driven shRNA is an effective and safe therapeutic in mice

In order to evaluate the therapeutic potential of our pol II promoter-dependent shRNA expression design, we engineered the liver-specific expression construct to suppress HBV in mice that were transgenic for the viral genome. This model system recapitulates the clinically relevant case of the potential infection of every hepatocyte in affected humans. For achieving viral suppression we selected a 25-mer shRNA with a 7-nucleotide loop targeting the HBV envelope surface antigen (sAg). While proving effective in target silencing, this hairpin was among the most potent inducers of hepatocellular toxicity when expressed from a U6 promoter.[6] We chose this shRNA specifically to test our hypothesis that restricting the expression to the liver at a reduced level would be better tolerated without compromising effective antiviral activity.

We packaged the ApoE/hAAT promoter-driven 3′-box-terminated shRNA (at +6 from the promoter) as a double-stranded DNA into pseudotyped AAV8, as in our previous study[6] (**Figure 2a**). Recombinant particles were then delivered by routine tail-vein injection at various doses to transgenic HBV mice, and their serum sAg levels were determined using enzyme-linked immunosorbent assay as a measure of HBV knockdown

(Figure 2b). At a dose of 3×10^{11}, silencing by the pol II construct was unstable, with serum sAg levels initially reduced by 80% from starting levels. After day 47 after injection, sAg levels rose slowly to 47% of starting levels by day 96. The highest dose, 1×10^{12} particles achieved a stable reduction in starting serum sAg of 84.5%, and this was subsequently verified directly by measuring viral genomes in sera, using quantitative PCR. According to these measurements, the starting levels of circulating genomes in this group were reduced by 93.8% at day 47 after injection, and by 85.4% at day 96.

Serum sAg levels also dropped in the negative control group that had received 3×10^{11} particles containing a double-stranded expression cassette for a 25-mer shRNA against Fluc, but this drop in levels was at a much lower rate (Figure 2b). This indicates the instability of the target in the model, and provides the rationale for the choice of end point. Also, because of this instability, the serum sAg levels of mice receiving the U6 promoter construct at 1×10^{12} particles were too low at the outset to allow for accurate measurement of silencing. Our previous work using this same viral construct showed an ~32-fold reduction in serum sAg within 2 weeks after a dose of 3×10^{11} particles; this was accompanied by substantial liver toxicity and the mice died shortly thereafter.[6] Although they did not show such rapid and dramatic silencing, the mice receiving the same hairpin under the liver-specific pol II promoter displayed no signs of toxicity whatsoever.

In order to confirm the absence of hepatocellular toxicity in the mice that received our novel construct, we quantified serum alanine aminotransferase (ALT) levels and compared them to those of positive control mice that received the original U6-expression construct with the same hairpin (Figure 3). In line with earlier reports,[6] the positive control mice exhibited a rapid rise in serum ALT levels, indicative of liver damage, and died after 3 weeks. In marked contrast, the serum ALT levels of mice that received the ApoE/hAAT expression cassette at doses of 3×10^{11} or even at 1×10^{12} dsAAV8 were stable and low throughout the 96-day period of measurement.

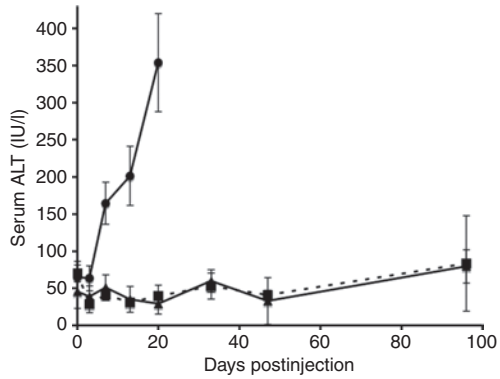

Figure 3 Comparison of liver toxicity levels between pol II and pol III shRNAs. Serum ALT levels in the same HBV mice as those referred to in **Figure 2** over a 96-day period. All constructs were delivered as dsAAV8 and express the same 25-mer shRNA against sAg. 1×10^{12} U6 promoter-driven (circles, $n = 3$), 1×10^{12} ApoE/hAAT promoter-driven (triangles, $n = 4$), and 3×10^{11} ApoE/hAAT promoter-driven (squares, $n = 3$). U6 group members all died at ~3 weeks. Error bars represent ±1 SD.

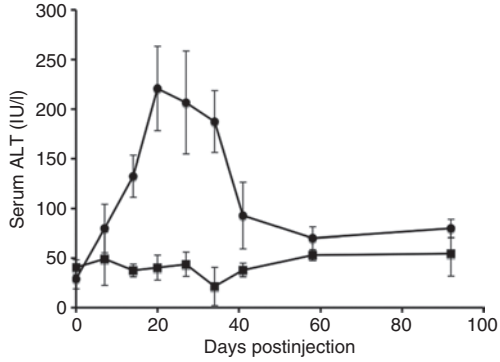

Figure 4 Comparison of liver toxicity levels in wild-type FVB mice. Serum ALT levels in wild-type FVB mice that received 3×10^{11} dsAAV8 of either the U6 promoter-driven (circles) or ApoE/hAAT promoter-driven (squares) 25-mer shRNA against sAg. For both injection groups $n = 6$. Error bars represent ±1 SD.

Comparison of toxicities in wild-type mice

As additional validation of the difference in toxicity profiles, we evaluated the effect of both promoter cassettes in the absence of an HBV background, in wild-type FVB mice. We again determined the serum ALT levels of mice that received either the U6 or ApoE/hAAT shRNA expression cassette at the moderate dose of 3×10^{11} dsAAV8 (Figure 4). Although shRNA expression from the U6 promoter was below the lethal threshold in this experiment, it was nevertheless toxic, as was evident from the rapid rise in serum ALT, with a peak at ~3 weeks and a gradual return to normal levels. As was observed in HBV mice, serum ALT remained normal in wild-type mice that had received the pol II ApoE/hAAT cassette, further confirming absence of toxicity, whereas the U6-expression cassette produced toxicity even at this moderate dose.

In order to compare the shRNA expression levels of these two cassettes, we probed total RNA extracted from the livers of mice at this dose. At 2 weeks, shRNA expression from the U6 promoter was significantly higher than from the ApoE/hAAT promoter (~40-fold by direct comparison on a single blot, data not shown). However, by week 6 after injection, detectable U6 transcripts fell to ~3% of their week-2 levels, and to <1% by week 14 (Figure 5a). Considering the spike and subsequent return to normal serum ALT levels, this drop in expression is consistent with the death of hepatocytes receiving the toxic cassette, and subsequent liver repopulation by nontransduced cells.[6] Expression from the ApoE/hAAT promoter, although lower at the first time point, remained stable throughout the first 14 weeks after injection. In one mouse in this group, the expression persisted at ~50% of peak levels even after one year (Figure 5b).

In order to rule out the possibility that a difference in initial transduction was the cause of the drastic difference in expression at 2 weeks after injection, we probed genomic DNA isolated from the same livers for the total number of vector genomes. In accordance with equal dosing and transduction rates, genome copy numbers were roughly equivalent for both groups at 2 weeks after injection (Figure 6a). On the basis of signal intensities observed for dilutions of plasmid DNA, the absolute values for samples at these time-points were between 100 and 140 copies per cell, approximately. The data at later time-points supported the observed trends in shRNA expression (Figure 5). The numbers of vector genomes containing the U6 promoter cassette fell sharply to undetectable levels at 6 weeks, while those with the

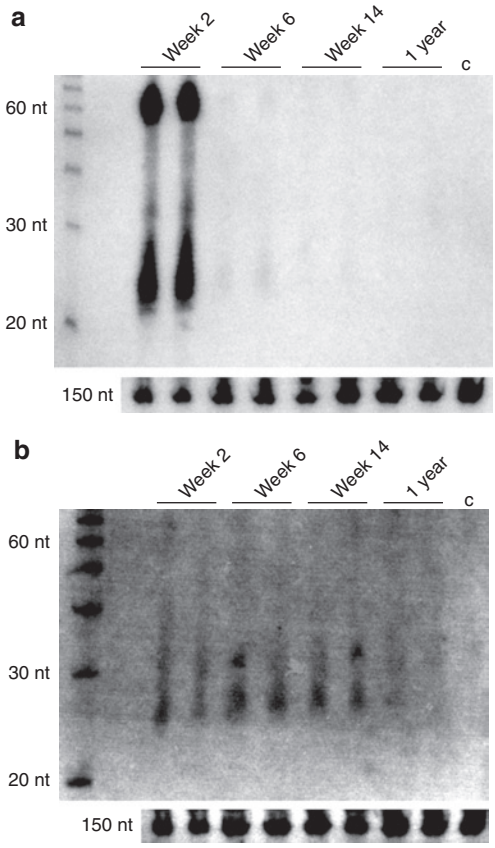

Figure 5 shRNA expression in wild-type FVB mouse liver. All mice (except untreated control "c") received a dose of 3×10^{11} dsAAV8, with two mice being killed at each of the indicated time-points. Each lane of the above small RNA Northern assays was loaded with 35 µg total liver RNA. The indicated sizes are marked according to the RNA ladder. The small panel beneath each blot shows a nonspecific band from the same blot, and hybridization, ~150 nt long, serving as internal loading control. (**a**) RNA from mice that received U6 promoter-driven 25-mer shRNA against sAg. (**b**) RNA from mice that received ApoE/hAAT promoter-driven 25-mer shRNA against sAg.

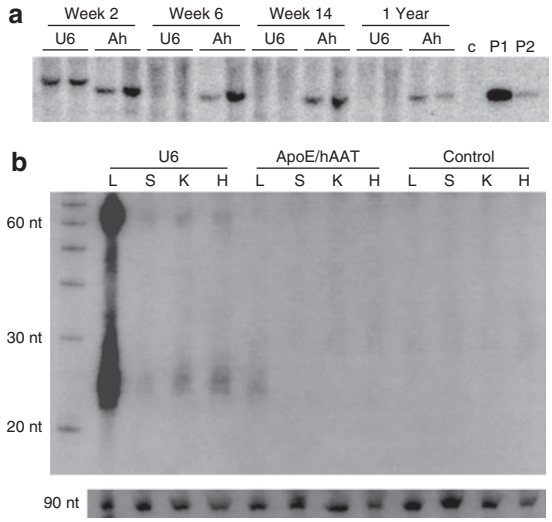

Figure 6 Vector abundance in liver and activity in various tissues. (**a**) Southern blot of 10 µg genomic DNA collected from livers of mice that received 3×10^{11} dsAAV8 of either U6 or ApoE/hAAT promoter-driven 25-mer shRNA against sAg. Labeled bands correspond to linearized vector genome monomers. The final three lanes contain liver DNA from an unmanipulated control mouse (c), and plasmid DNA representing ~444 (P1) and 44.4 (P2) vector genome copies per cell. (**b**) Northern blot of 30 µg total RNA collected 2 weeks after injection from liver (L), spleen (S), kidney (K), and heart (H) of a mouse that received either 3×10^{11} (U6) or 1×10^{12} (ApoE/hAAT) dsAAV8. The small bottom panel shows a nonspecific band from the same blot, and hybridization, ~90 nt long, serving as internal loading control.

ApoE/hAAT promoter persisted at a stable level for more than 3 months. Even after one year, pol II vector genomes remained at ~50% of week-2 levels.

Finally, we sought to verify the expected tissue specificity of our ApoE/hAAT shRNA expression construct. Wild-type FVB mice were injected with either 3×10^{11} of the U6 or 1×10^{12} of the ApoE/hAAT dsAAV8 vector. Total RNA was isolated from livers removed from the animals at 2 weeks after injection, when the U6 shRNA expression cassette was observed to still be active (**Figure 5a**), as well as from three major nonhepatic targets for AAV8 transduction—spleen, kidney, and heart.[24] Expression of shRNA from the ubiquitously active U6 promoter was detected in all tissues by northern blot (**Figure 6b**). Despite a vector dose more than three times higher, shRNA expression from ApoE/hAAT was not detected in any tissue aside from liver, even with extensive overexposure of the blot (data not shown). While this does not necessarily rule out activity at a level below the detection threshold, it would nonetheless be dramatically lower than those of the clearly detectable U6 transcripts, and carry a commensurate reduction in promiscuous RNAi activity in nontarget tissues.

DISCUSSION

In this study, we have described the optimization and therapeutic evaluation of a system for liver-specific RNAi induction *in vivo*. This system allows for more precise control while also being designed to maintain a minimal hairpin structure. Moreover, there are three important differences between this system and others that employ pol II promoter transcripts for RNAi induction. These are: (i) The shRNA is not embedded within additional sequences for miRNA mimicry. This simplifies cloning efforts and reduces the risk of setting up a competition with endogenous miRNA for processing by the Drosha/DGCR8 complex.[15] (ii) While there are some limited examples of nonmimic shRNAs expressed from pol II promoters,[25] the hairpins described here were thoroughly optimized, with specific attention being paid to maximally functional minimal overhangs. (iii) Tissue specificity and silencing activity are achieved without the need for additional elements.[26]

In addition to providing confirmation of previous observations concerning the detrimental effects of long shRNA 5′-overhangs on RNAi induction,[17] we also observed that a slight negative effect was produced when an shRNA was placed at +1 from a pol II promoter. This may be attributable to shifting the initiation site to transcribe an shRNA with less favorable kinetics of active-strand incorporation into RISC.[27,28] In that case, the negativity of this shift would be sequence-dependent and may not be an issue in other shRNAs. The fact remains that swapping sequences is far easier with initiation at +6 to +7 after a restriction site, an arrangement that we have validated as effective with two distinct shRNAs (luciferase and HBV sAg). While the detrimental effects of further reduction may be peculiar to the sequence we have used in this

study, there is at least no inherent advantage in achieving further shortening (although a means of accomplishing this has also been suggested).[29]

Constructs involving the U1 3′ termination box in place of the previously published minimal polyA signal were employed initially in order to continue the trend toward shorter 3′-shRNA-overhang; however, the slight but distinct increase in shRNA activity from such constructs served as another step toward optimization. One possible explanation is that the 3′-box enhances the processing of the transcribed shRNAs into siRNAs. The relatively low prevalence of these primary pol II shRNA transcripts and, presumably, the rapid processing, are reflected in the complete lack of signal corresponding to the shRNA form observed for U6 transcripts. Low prevalence is an obstacle to traditional means of manipulation, but high-throughput sequencing platforms may offer an insight into defining the 5′- and 3′-ends of these pol II shRNAs. These would provide valuable information for the further optimization of our vector design. Also unknown is the identity of the ~31 nt bands present in some RNA samples from mice that received the pol II shRNA expression cassette (**Figure 5b**). It may merely be a partially processed shRNA, such as ones observed earlier by others.[26]

Last but not the least, another crucial benefit of our novel shRNA expression system is the striking reduction in toxicity, thereby accommodating the use of high viral particle doses (*e.g.*, 1×10^{12} in mice). This results in a significant increase in the size of the therapeutic window for viral vector-mediated RNAi applications, permitting complete transduction of entire organs (here, the liver) without causing harm. Further reduction in U6 vector dose below those shown to induce toxicity and vector loss may come only at the expense of complete transduction, and this would be particularly problematic for an antiviral therapeutic. However, one must bear in mind the level of RNAi activity required for a given application and decide whether the sacrifice of shRNA expression levels is justified by the increase in control. Where an additional order of magnitude of target silencing is not required, or where concern for toxicity is more prominent, pol II shRNA expression systems such as this could prove to be valuable additions to our therapeutic repertoire.

MATERIALS AND METHODS

Constructs and cloning. All constructs have a pBluescript II KS(+) (Stratagene, La Jolla, CA) vector backbone, except U6 promoter constructs, as described in the later text. The ApoE/hAAT promoter (Ah), containing the hepatic control region of the human *ApoE* gene enhancer and the human α-1-antitrypsin promoter, was PCR-amplified from the CM1 plasmid[30] with upstream XbaI/NheI sites and an Nde1 site beginning at +1 (replacing a blunted PstI site in the backbone). The human ubiquitin C promoter was similarly cloned from UB6/V5-His B (Invitrogen, Carlsbad, CA), with (UbL) or without (UbS) intronic/exonic elements. Promoters were initially inserted into Bluescript by PstI-blunt/XbaI digestion. Subsequent promoter exchanges were by XbaI/NdeI digestion.

The U1 3′-box was added by EcoRI/HindIII digestion and by ligating two phosphorylated and annealed oligos, 5′-aattcgtttcaaaagtagagcggcc gca-3′ and 5′-agcttgcggccgctctacttttgaaacg-3′. These oligos did not include additional sequences from the 3′-end of the U1 snRNA, as they were found to be unnecessary (data not shown). The same procedure was followed for insertion of the previously described minimal polyA.[17] Finally, the hairpin targeting Fluc was added by ligating two phosphorylated and annealed oligos into NdeI/EcoRI sites, 5′-tatgggattccaattcagcgggagccacc tcaagagggtggctcccgctgaattggaatccg-3′ and 5′-aattcggattccaattcagcgggagcc accctcttgaggtggctcccgctgaattggaatccca-3′. The resulting constructs were designated AhlucSb (for ApoE/hAAT), UbSlucSb or UbLlucSb (for UbiC), and UbSlucpA (for minimal polyA). Removal of 5′ shRNA overhangs was accomplished with the use of the above oligos lacking the NdeI site, and the combination of hairpin and terminator sequences in a single oligo pair (PAGE-purified). The promoter and hairpin were ligated at a blunt-end junction while directionally cloned into Bluescript.

The random hairpin control replaced the shRNA template of UbSlucSb with phosphorylated and annealed oligos of matched GC-content, 5′-tatgcgtagaccctagatgaccagcgcagtcaagagctgcgctggtcatctagggtcta cgg-3′ and 5′-aattccgtagaccctagatgaccagcgcagctcttgactgcgctggtcatctagggtc tacgca-3′. For targeting sAg, the shRNA template sequence of AhlucSb was replaced with phosphorylated and annealed oligos, 5′-tatgaacaaatggcactag taaactgagtcaagagctcagtttactagtgccatttgttcg-3′ and 5′-aattcgaacaaatggcacta gtaaactgagctcttgactcagtttactagtgccatttgttca-3′. When the initial guanine of the hairpin was considered as the last base of NdeI, this placed the hairpin at +6 from the promoter. The entire expression cassette was then PCR-amplified for insertion into an AAV packaging plasmid containing ITRs for packaging as a double-stranded DNA and human alphoid repeats to raise the total packaging size to ~2 kb. Constructs expressing shRNA from a U6 promoter and the dsAAV8-packaged cassette expressing the 25-mer shRNA against sAg are as previously described.[6]

Dual-luciferase assays. Mouse hepatoma Hep1A cells were seeded at $1.5–1.6 \times 10^5$ cells per well in 6-well plates. One day later, cells at ~30% confluence were transfected with plasmid DNA in complex with SuperFect (Qiagen, Valencia, CA), in accordance with the manufacturer's recommendations. All wells received 0.2 μg pGL3, with pRL-SV40 (Promega, Madison, WI) in a molar ratio of 25:1 pGL3:pRL. Each group, comprising three wells each, also received fivefold pGL3-molar-equivalents of the indicated shRNA vector. The dual-luciferase assay (Promega) was then conducted 48 hours after transfection as directed, using an LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Hydrodynamic transfection assays. All animal procedures were conducted in accordance with the guidelines relating to animal care at Stanford University. BALB/c female mice aged 8.5 weeks (Jackson Laboratory, Bar Harbor, ME) were hydrodynamically transfected with 2 μg RSV-hAAT, 2 μg pGL3, and 5 pGL3-molar-equivalents of the indicated shRNA vector in 1.8 ml 0.9% NaCl (5 mice per group). On day 3 after the injection, the mice received 1.5 mg D-luciferin (Xenogen, Alameda, CA) in 100 μl Dulbecco's phosphate buffered saline by IP injection, and were imaged 10 minutes later using an IVIS 100 imaging system (Xenogen). Readings were normalized to hAAT levels measured in serum collected on day 4, so as to adjust for transfection efficiency.

Viral vector packaging, titering, and injection. Stabilized dsAAV8 was produced by triple-transfection and CsCl purification as previously described.[6] Titers were determined by dot blot. The virus was diluted as required to 300 μl in Dulbecco's phosphate buffered saline. Viral dilutions were delivered by routine tail-vein injection.

Mice. Wild-type BALB/c (for *in vivo* imaging) and FVB (for viral delivery) mice were procured from Jackson Laboratory. FVB mice carrying an integrated copy of the HBV genome (STC lineage) have been described earlier.[16]

Serum collection and analysis (enzyme-linked immunosorbent assays and ALT). Enzyme-linked immunosorbent assays were performed using dilutions of sera from blood collected retroorbitally with nonheparinized capillary tubes, using standard methods. After dilution with phosphate buffered saline containing 10% fetal calf serum, serum sAg concentrations were measured using the Auszyme Monoclonal Diagnostic ELISA kit

(Abbott Laboratories, Abbott Park, IL). Serum ALT levels were measured using colorimetric assay, in accordance with the directions given (Teco Diagnostics, Anaheim, CA).

Tissue collection and DNA/RNA isolation. Collected tissues were sectioned as needed, immediately frozen in liquid nitrogen, and stored at −80 °C prior to use. RNA was extracted from homogenized tissue using TRIzol (Invitrogen) (including an additional acid-phenol purification step), and precipitated overnight at −20 °C. DNA was extracted from homogenized tissue by proteinase K and subsequent LiCl treatment.

Northern and Southern blotting. Samples of total RNA were run on 15% acrylamide 7 M urea denaturing gels. Samples were blotted and probed using standard methods. The DNA oligo probe used for the U6 and multi-tissue Northern assays is complementary to the first 19 bases of the antisense arm of the hairpin against sAg, 5′-ttactagtgccatttgttcga-3′. For greater sensitivity in detecting the weaker pol II shRNA signal, an optimized version of the first probe with 21 bases of complementarity (5′-gttt actagtgccatttgttc-3′) was used for the ApoE/hAAT Northern assay. In all cases, equal loading was verified by ensuring equal intensities of a non-specific band common to all the samples (including liver RNA from an unmanipulated mouse) resulting from the same hybridization.

For the vector genome Southern blot, 10 µg of liver genomic DNA was digested with *Bgl II* to linearize the vector genomes as monomers, run on an agarose gel, and blotted using standard methods. Dilutions of plasmid DNA were likewise digested. The DNA oligo probe is complementary to the first 25 bases of the shRNA template (5′-ctcagtttactagtgccatttgttc-3′), the only DNA sequence that is common to the U6 and Ah constructs.

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