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RNA polymerase III can drive polycistronic expression of functional interfering RNAs designed to resemble microRNAs

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ABSTRACT

In both research and therapeutic applications of RNA interference, it is often advantageous to silence several targets simultaneously. Toward this end, several groups have developed vectors that utilize the model of endogenously encoded micro (mi) RNAs, where a single RNA polymerase II promoter can drive the expression of multiple interfering RNAs. Stronger pol III promoters have been used to drive individual short hairpin (sh) RNAs, but to date, it has been necessary to repeat the promoter in each silencing cassette to achieve multiplexed expression from a single vector. Here, we show that it is possible to drive polycistronic expression from a single pol III promoter when the interfering RNAs are formatted to resemble miRNAs rather than shRNAs. As many as four miRNAs designed to target hepatitis B virus (HBV) transcripts are shown to be processed and functional in reporter assays as well as in the context of replicating virus in cell culture systems. Although it has been observed that high levels of expression of shRNAs can lead to cytotoxicity, we find no significant evidence in transient transfection assays that the HBV-miRNAs produced by our vectors compete for the activity of endogenously produced miR-122 or for processing of an exogenously expressed miR-EGFP.

INTRODUCTION

The development of technologies that use RNA interference (RNAi) to control gene expression has had a large impact in numerous areas of research and has provided opportunities for new therapeutic strategies in the treatment of genetic and infectious diseases. For many applications, it is proving advantageous to utilize multiple interfering RNAs that can simultaneously target different gene products. This is particularly true in the development of RNAi-based antiviral therapeutics, where the use of several interfering RNAs can help to induce strong suppression of viral replication across a broad range of naturally occurring genotypic variants as well as to suppress the emergence of escape mutants [reviewed in (1)].

A number of different methods have been used to generate multiple interfering RNAs to suppress viral replication. A mixture of several short interfering (si) RNAs, produced exogenously by chemical synthesis or by endonuclease III treatment of longer double-stranded RNAs, has been effective against hepatitis C and B virus (HCV and HBV) and can help reduce viral escape in cell culture and animal models of infection (2–4). Multiple siRNAs can also be produced by intracellular dicing of long hairpin (lh) RNAs with stem structures as long as 50–100 bp. By introducing base changes into the sense strand of these intracellularly transcribed lhRNAs, to partially disrupt their double-stranded structure, it was possible to retain silencing activity against HCV (5) or HBV RNAs (6) without inducing an interferon response. It has also been demonstrated that multiple anti-HIV siRNAs that target noncontiguous sites can be processed from a pol III driven lhRNA, although silencing activity derived predominantly from the siRNA encoded closest to the base of the hairpin (7).

Additional approaches for multiplexed silencing have utilized plasmid or viral vectors capable of expressing multiple interfering RNAs that are formatted as short hairpin (sh) or micro (mi) RNAs. Often, pol III promoters are used to express shRNAs, sometimes together with other RNA-based effectors such as decoys or antisense RNAs, that silence viral transcripts or cellular RNAs necessary for viral replication. This strategy has shown good success against viruses including HIV-1, HCV and HBV that otherwise would readily escape the efficacy of single shRNAs (8–12). In all cases, however, a separate promoter

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has been used for each RNA-based effector in the multiplexed vector and this can be problematic. For instance, the repetition of H1 promoters in an anti-HIV lentiviral vector led to a high rate of recombination and deletion of the shRNA expression cassettes, diminishing the vector's antiviral efficacy and resistance to escape (13). To avoid this problem, it was necessary to use a different pol III promoter to drive the expression of each shRNA (13). Alternatively, multiple interfering RNAs that are designed to resemble endogenous miRNAs can be processed from a single RNA pol II transcript. The efficacy of pol II driven polycistronic antiviral miRNAs has been demonstrated for HIV-1 and HBV (14–17).

The use of either pol II or pol III promoters to drive expression of silencing cassettes each brings both advantages and disadvantages, and different modes of expression will be appropriate in different applications. In general, pol II promoters can be utilized for cell type specific expression or regulation and for efficient expression of more than one interfering RNAs from a single transcript. On the other hand, pol III promoters are ubiquitously expressed and stronger, potentially leading to more potent silencing from low copy number vectors. While it has been shown both theoretically (18) and experimentally (12,13) that viral escape is reduced by strong, multiplexed silencing, it is also true that high expression levels of shRNAs can be toxic in cell systems and in animals (19–21). Evidence is emerging, however, that miRNAs are better tolerated and less likely to induce toxic effects (21–24). In the work described here, we have explored whether a single pol III promoter can be used to drive the expression of multiple miRNA-formatted interfering RNAs, thereby combining the advantages of multiplexed silencing from a compact and nonrepetitive expression cassette with the strength of a pol III promoter and the lower toxicity of the miRNA configuration.

MATERIALS AND METHODS

Plasmid constructions

We have previously described the construction of plasmids that encode RNA pol II-driven multiplexed miRNAs designed to target different regions of HBV transcripts (15). In the experiments described here, the Xba I/Spe I fragment of pLV-30s/1737B/1907A, which encodes 1737B and 1907A HBV-miRNAs, was PCR amplified using a forward primer (5′-CTAGGTCGACCACTATTATTTC TATCGTCTAGAAGGC-3′) that adds a Sal I site and retains the Xba I site at the 5′-end of the amplicon, and a reverse primer (5′-GGTACCAAAAAACGGCTGCTG AATCGACTAGTAGCC-3′) that retains the Spe I site and adds a $(T)_6$ termination signal followed by a Kpn I site at the 3′-end. (Restriction sites are underlined in the primer sequences.) This Sal I/Kpn I fragment was inserted immediately downstream of a pol III promoter in a pUC-19-based plasmid where the promoter is flanked by 5′-Pst I and 3′-Sal I restriction sites. The 2791B HBV-miRNA Xba I/Spe I fragment was removed from a monocistronic plasmid and inserted into either the Xba I site or the Spe I site to generate tricistronic plasmids. The 2910A HBV-miRNA fragment was assembled from oligonucleotides as previously described (15) and inserted into the Xba I site or the Spe I site of a tricistronic plasmid to generate polycistronic plasmids encoding four HBV miRNAs in the order 1737B/1907A/2791B/2910A or 2910A/2791B/1737B/1907A (Figure 1). The 2910A target sequence is TCGTGGTGGACTTCTCTCAATT near position 2910 in the HBV (strain ayw) genome (GenBank accession number V01460.1), and other target sequences are reported in (15). The human U6 promoter was amplified from pUC-U6-30/EGFP using a forward primer (5′-AAACTGCAGGATCCCCCCGAGTCCA-3′) to add a Pst I site at the 5′-end and a reverse primer (5′-CCTA GTCGACTGGTATATGTGCTGCC-3′) to add a Sal I site and destroy the Spe I site at the 3′-end of the promoter. All constructions were confirmed by DNA sequencing.

The HBV infectious molecular clone, pHBV/2 and psiCHECK-2 dual luciferase reporter vectors containing HBV target sequence have been described (15). The reporter plasmids psiCH-CAT360 and psiCH-CAT1454 contain, respectively, 360 and 1454 bp of sequence encoding the 3′-UTR of human cationic amino acid transporter (CAT-1) mRNA inserted into the Renilla luciferase 3′-UTR in psiCHECK-2. These regions correspond to those included in luciferase reporter plasmids RL-catC (for CAT360) and RL-catB (for CAT1454) constructed and characterized by Bhattacharyya *et al.* (25). The CAT360 sequence contains no predicted targets for miR-122 and the CAT1454 sequence contains three predicted targets (25). [Region D that confers stress-mediated regulation (25) is not included in these plasmids.] For psiCH-CAT360, the forward primer 5′-CATACTCGAGCACAG CCCCGCCCCCCG-3′ was used with the reverse primer 5′-TAGCGGCCGCGGAAGGCCTGGTTCC-3′ for PCR amplification of the CAT-1 3′-UTR from a cDNA clone obtained from Open Biosystems (ID 30340706) with subsequent cloning into Xho I and Not I sites in psiCHECK-2. For psiCH-CAT1454, the reverse primer 5′-TAGCGGCCGCCATCAGCATGGGCCTTC TGTCTCCT-3′ was used. The primers were based on those previously reported (25). All constructions were confirmed by DNA sequence analysis.

Cell culture

Human hepatoblastoma Huh7 and HepG2 cells were grown in RPMI 1640 supplemented with L-glutamine, 25 mM HEPES and 10% FBS. HEK-293T cells were grown in DMEM supplemented with 4.5 g/l glucose, L-glutamine, sodium pyruvate and 10% FBS. All cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Silencing assays

For dual luciferase reporter assays of silencing, Huh7 cells were plated in 12-well plates and transfected using Arrest-In (Open Biosystems, Huntsville, AL) according to the manufacturer's directions. The quantity of target and effector plasmids used in each transfection is indicated

in figure legends. Two days posttransfection, cells were lysed and firefly and Renilla luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI).

To assess silencing in the context of viral replication, Huh7 cells were plated and transfected with pHBV/2 together with polycistronic silencing plasmids and the reporter plasmid pGluc (New England Biolabs, Beverly, MA, USA), as indicated in the legend of Figure 5. Two days posttransfection, culture supernatants were harvested and assayed for secreted HBV surface antigen (HBsAg) using Genetic Systems HBsAg EIA 3.0 (Bio-Rad Laboratories, Hercules, CA, USA) and for Gluc activity using a Gaussia luciferase Assay Kit (New England Biolabs).

Northern blot analysis of miRNA expression and processing

HEK-293T cells were plated in six-well plates and transfected with 250 ng HBV-miRNA expression plasmid (plus 1.75 μg pUC19 DNA, added as filler) by calcium phosphate mediated transfection. Cells were collected 48 h posttransfection and total RNA was isolated using a mirVana RNA isolation kit (Ambion, Austin, TX, USA). For each sample, 10 μg of RNA was separated by electrophoresis in a 12% polyacrylamide urea-TBE gel and then electroblotted to BrightStar Plus nylon membrane (Ambion). Blots were vacuum dried at 60°C for 1 h, prehybridized at 37°C for 1 h in ULTRAhyb-Oligo hybridization buffer (Ambion) and then ^{32}P-radiolabeled oligonucleotide probe was added for overnight hybridization at 37°C. Blots were washed 3 times in 2× SSPE/0.1% SDS at room temperature. Radiolabeled probes were made by kinase end-labeling of 21 nt oligonucleotides complementary to the antisense (guide) strand of mature HBV-miRNAs. Probe for U6 RNA was made by random-primed radiolabeling of a 127 bp DNA fragment generated by PCR of human DNA. Hybridization was detected using a Storm 820 (GE Healthcare) phosphorimager. Blots were stripped by boiling briefly in 0.2× SSPE/0.1% SDS prior to hybridization with a different probe.

Immunoblot analysis

Cells were harvested 2 days posttransfection, washed in PBS and lysed in 0.5× RIPA buffer with added complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). After electrophoresis in a 12% polyacrylamide–SDS gel, proteins were transferred to PVDF membrane and probed with α-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-EGFP (Chemicon, Temecula, CA, USA) antibodies. Secondary binding of horseradish peroxidase conjugated anti-rabbit antibody (Sigma, St Louis, MO, USA) was detected using Super Signal West Dura Extended reagent (Pierce, Rockford, IL, USA) and imaged with a FluorChem SP digital camera (Alpha Innotech, San Leandro, CA, USA).

RESULTS

Silencing activity from multiplexed HBV-miRNAs driven from pol III promoters

In previous work, we have described vectors that use RNA pol II promoters to express up to four different HBV-miRNAs from a single polycistronic transcript (15). In these vectors, the HBV-miRNAs are encoded in the context of hsa-miR-30, including flanking sequence of ~40 bp 5′ and 30 bp 3′ of each stem-loop structure. The flanking sequence allows excision of the HBV-miRNAs from the primary transcript as pre-miRNAs with further processing to mature interfering RNAs via the cellular pathway used for the generation of endogenous miRNAs. To date, RNA pol III promoters have been used by many investigators to express shorter and less complex RNAs, such as shRNAs or individual miRNAs, where interfering RNAs can be processed from a single stem-loop. However, a recent report showing that miRNAs can be found encoded downstream of some Alu repeats and can be processed from the highly structured pol III Alu transcripts (26) prompted us to test whether our multimerized HBV-miRNAs could also be expressed from a pol III promoter. As outlined in Figure 1, DNA encoding either two or four individual HBV-miRNAs from our original pol II driven constructs [(15), and see 'Materials and Methods' section] was recloned downstream of a pol III promoter (either U6 or H1) in a pUC-based plasmid. Silencing activity of these plasmids was tested in Huh7 cells, using a dual luciferase assay based on the reporter vector, psiCHECK2. The reporter plasmid psiCH-HBV has the entire HBV genome inserted into the 3′-UTR of the Renilla luciferase expression cassette in psiCHECK2 such that transcripts contain target regions for all of the HBV-miRNAs, as previously described (15). Firefly luciferase is expressed independently from the same plasmid and serves as a transfection efficiency control. Cells were transfected with a constant amount of psiCH-HBV reporter plasmid together with increasing amounts of each silencing plasmid. Results shown in Figure 2a indicate that plasmids encoding either two or four HBV-miRNAs are able to silence the psiCH2-HBV reporter. Further, plasmids that use the U6 promoter are more potent than those that use the H1 promoter, as expected based on the relative strength of these promoters (19,27). When directly compared with our previously reported pol II driven polycistronic HBV-miRNA expression plasmids, efficacy is again related to the predicted strength of the promoters, as shown in Figure 2b. For instance, using 10 ng of a bicistronic plasmid, the LV (liver specific) and CMV pol II promoter-driven effector plasmids silence reporter expression to 70.2 and 44.1% of control levels (no added effector plasmid), while plasmids with the stronger H1 and U6 pol III promoters direct silencing to 17.7 and 8.5% of controls, respectively. Expression of miRNAs directed against EGFP had no effect on the HBV reporter.

Interestingly, plasmids that express four HBV-miRNAs show slightly less silencing activity compared to plasmids that express only two HBV-miRNAs from the same pol III promoter (pUC-U6-2 versus pUC-U6-4 and pUC-H1-2 versus pUC-H1-4 in Figure 2a). This effect

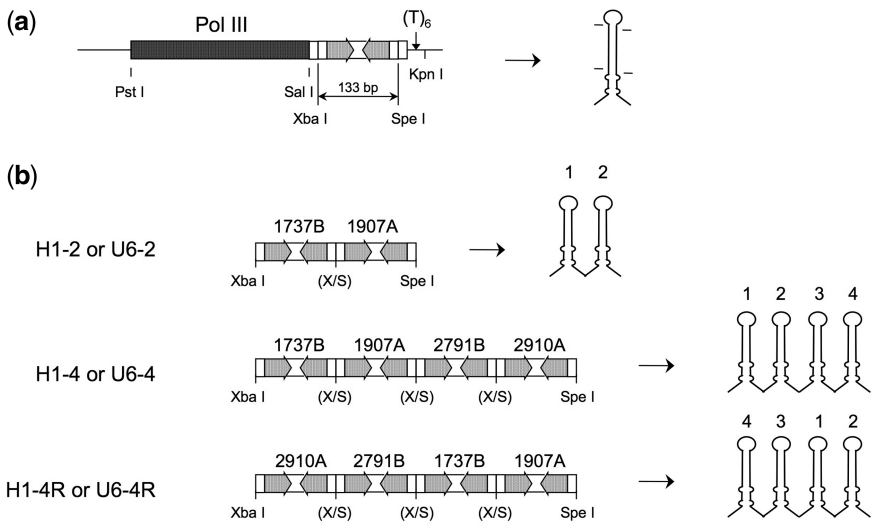

Figure 1. Organization of cassettes for RNA pol III driven expression of miRNAs. (**a**) General structure of an expression cassette with a pol III promoter and restriction sites for insertion of DNA encoding a pre-miRNA. Facing block arrows (light gray) represent DNA encoding the stem portion and open bars show the flanking and loop sequences for each pre-miRNA. The shaded bar (dark gray) shows the pol III promoter and the $(dT)_6$ pol III termination signal is indicated. The predicted structure of the pre-miRNA transcript is shown at the right, including sequence from the hsa-miR30 flanking regions that form a partially base paired structure at the base of the stem-loop necessary for recognition by the Drosha/DGCR8 microprocessor complex (40). Hash marks next to the stem-loop show predicted cleavage positions for processing to mature miRNA. (**b**) Structure of DNA encoding the bicistronic and polycistronic HBV-miRNAs and their RNA transcripts. DNA encoding the 1737B/1907A pre-HBV-miRNAs was inserted downstream of a pol III H1 or U6 promoter in a pUC-based plasmid to produce the bicistronic H1-2 and U6-2 silencing plasmids. Sequential insertion of XbaI/SpeI fragments encoding individual 2791B or 2910A pre-HBV-miRNAs produced the polycistronic cassettes H1-4 and U6-4. The position of insertion of individual HBV pre-miRNAs was changed to generate the rearranged cassettes, H1-4R and U6-4R. HBV-miRNAs are numbered according to their target site in the HBV genome. The expected structure of transcripts from each cassette is shown at the right, with individual HBV-miRNA stem-loops numbered (1–4) to highlight their order in the primary transcript.

can neither be completely accounted for by the slight difference in size of the plasmids (~7%) nor by saturation of processing pathways, since the amount of silencing still increases with plasmid dosage and promoter strength. Instead, this may be a reflection of less than optimal transcription, folding or processing of the longer quadraplexed primary miRNA transcript, although potent silencing activity is clearly retained for these plasmids.

Functional testing of individual HBV-miRNAs from the polycistronic expression cassettes

The silencing activity of each miRNA encoded in the polycistronic plasmids was tested using psiCH2 dual luciferase reporter plasmids that contain insertions of target sequence for each of the individual HBV-miRNAs. Huh7 cells were transfected with a reporter plasmid carrying a single target region, together with a polycistronic silencing plasmid (pUC-U6-2 or U6-4, or pUC-H1-2 or H1-4). For comparison, monocistronic plasmids with single HBV-miRNAs expressed from a U6 promoter were also cotransfected with reporter plasmids carrying their cognate target. Results shown in Figure 3a indicate that each of the HBV-miRNAs is roughly as active in the context of polycistronic expression as it is when expressed from a monocistronic silencing plasmid. We again see somewhat less activity from plasmids that encode four miRNAs as compared to those with two, and stronger activity from the U6 plasmids than from the H1 plasmids. These results support the conclusion that the overall silencing potency of the polycistronic plasmids observed in Figure 2 reflects the combined effects of each component miRNA, and not an activity dominated by a single miRNA.

The silencing activity of the 2910 HBV-miRNA was weak at the low dose tested (Figure 3a) whether it was expressed alone from a monocistronic plasmid or as the most distal stem-loop in a polycistronic plasmid. Nevertheless, this result raised the question as to whether pol III promoters are capable of expressing as many as four miRNAs or lose activity over the span of several stem-loop structures. To address this, we constructed plasmids where the order of the four HBV-miRNAs was rearranged, placing 2910 in the first position, followed by 2791, 1737 and 1907 (Figure 1b). Figure 3b shows results of a transfection experiment comparing the activity of individual HBV-miRNAs against their cognate target sequence when they are placed in a different order in the polycistronic silencing plasmid (pUC-U6-4 versus pUC-U6-4R). Here, it is evident that moving 2910 and 2791 miRNAs to the first and second positions dramatically improves their potency. At the same time, 1737 and 1907 miRNAs did not lose any potency when encoded in more distal positions. While a full explanation of this effect needs further investigation, we can conclude that pol III is able to transcribe at least four miRNAs. We note that there are no stretches of greater than three T residues that could lead to transcriptional termination in the HBV-miRNA coding region of any of our plasmids. It seems likely that, depending on the sequence of each of the component miRNAs, changes in their position can

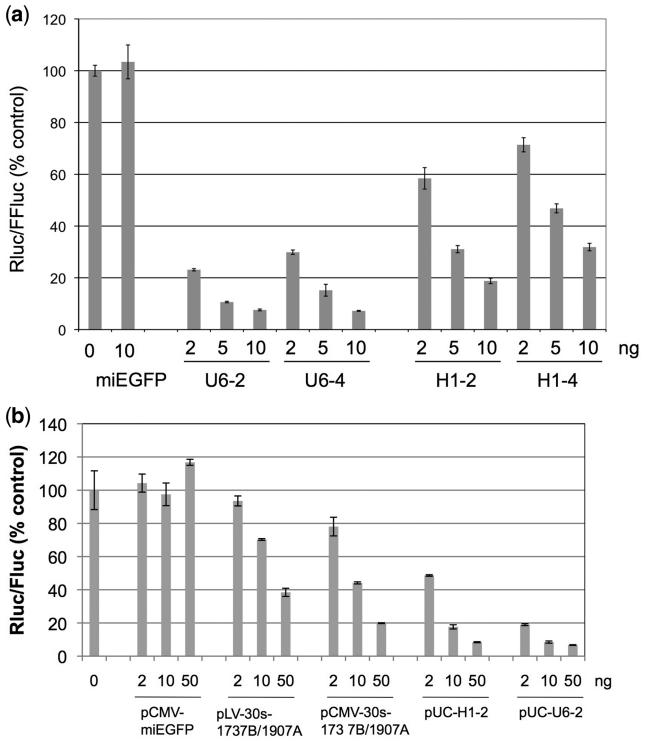

Figure 2. Silencing activity of polycistronic miRNA constructs. (**a**) Huh7 cells were transfected with 250 ng of a dual luciferase reporter plasmid, psiCH-HBV, which contains sequence targeted by all four of the HBV-miRNAs, together with no silencing plasmid or increasing amounts of plasmids carrying the U6-2, U6-4, H1-2 or H1-4 HBV-miRNA expression cassettes, as indicated. Cotransfection with the reporter plasmid and 10 ng of a U6-miEGFP silencing plasmid (miEGFP) served as a negative control. Silencing activity was measured as the ratio of Renilla to firefly luciferase activity in cell lysates 2 days posttransfection, and results are reported as percent of control with no added silencing plasmid. Data represent the average (± SD) of triplicate assays of two separate transfections. (**b**) Huh7 cells were transfected with plasmids expressing two HBV-miRNAs from a pol II promoter [pCMV-30s-1737B/1907A and pLV-30s-1737B/1907A, see (15)] or the corresponding pol III driven cassettes (pUC-H1-2 and pUC-U6-2) and analyzed as in (a).

result in altered folding and processing of the primary transcript, as noted earlier.

Detection of mature miRNA derived from each of the multiplexed HBV-miRNAs

A further test of the expression of all four HBV-miRNAs was performed by northern blot analysis to detect mature miRNAs produced from the polycistronic plasmids. HEK-293T cells were transfected with U6 driven monocistronic plasmids expressing each of the individual HBV-miRNAs or with the pUC-U6-4, pUC-H1-4 or pUC-U6-4R polycistronic plasmids. RNA isolated from the transfected cells was analyzed by sequential hybridization of blots using radiolabeled probes to detect each of the HBV-miRNAs, or U6 RNA as a loading control. Figure 4 shows that similar mature miRNAs can be detected for each of the HBV-miRNAs when they are expressed from mono- or polycistronic plasmids.

Silencing of HBV RNAs in the context of viral replication

In order to confirm that the plasmids can silence HBV transcripts in the context of viral replication, the pUC-U6-4 and pUC-U6-4R plasmids were tested by cotransfection with a plasmid that produces infectious HBV. Huh7 cells were cotransfected with pHBV/2, increasing amounts of pUC-U6-4 or pUC-U6-4R, and pGluc. Two days posttransfection, cell supernatants were assayed for secretion of HBsAg as an indicator of viral replication and Gaussia luciferase as a transfection efficiency control. Normalized HBsAg levels, as shown in Figure 5, indicate that the polycistronic plasmids are potent inhibitors of viral replication. The re-arranged order of HBV-miRNAs found in pUC-U6-4R leads to stronger overall silencing as expected from the higher silencing activity of the individual miRNAs, as shown in Figure 3b.

The endogenous miRNA pathway is not saturated by pol III expression of polycistronic HBV-miRNAs

Concerns have been raised about potential toxic effects from high levels of exogenous expression of interfering RNAs due to competition for components of the endogenous miRNA processing pathway (19–22). As an initial test of whether expression of the multiplexed HBV-miRNAs from a pol III promoter can saturate miRNA pathways, we looked for changes in the silencing activity of endogenously expressed miR-122 in transfected Huh7 cells. A psiCHECK-2 reporter with a single, perfectly matched miR-122 target sequence inserted into the 3′-UTR of the Renilla luciferase gene was used in these assays. In order to demonstrate miR-122-directed silencing of this reporter, we used it to transfect both Huh7 cells, which have high levels of miR-122, and HepG2 cells, which do not express miR-122. Results shown in Figure 6a confirm that the psiCH2-122 reporter is strongly suppressed in Huh7 cells, but not in HepG2 cells. The psiCH2-122 reporter plasmid was then transfected into Huh7 cells together with increasing amounts of pUC-U6-2, pUC-U6-4 or pUC-H1-4. Cells were also transfected with the psiCH2 plasmid, with no target, as a control for luciferase expression. When cells were assayed 2 days posttransfection, as shown in Figure 6b, there was no significant decrease in the miR-122 directed silencing of the reporter plasmid in the presence of any of the effector plasmids, even at doses five times higher than required for potent silencing of HBV targets (Figure 2).

We also tested whether expression of the HBV-miRNAs can cause a reduction in the activity of endogenous miR-122 against incompletely matched, validated targets found in the 3′-UTR of human CAT-1 mRNA (25,28). The dual luciferase reporter constructs psiCH-CAT360 and psiCH-CAT1454 contain sequence derived from the first 360 nt or 1454 nt of the CAT-1 3′-UTR, and correspond, respectively, to the RL-catC and RL-catB reporter plasmids of Bhattacharyya *et al.* (25). The CAT360 3′-UTR does not contain predicted miR-122 target sites and the CAT1454 3′-UTR contains three predicted targets (25). Despite the absence of miR-122 in HepG2 cells, normalized Renilla luciferase activity (Rluc/Fluc)

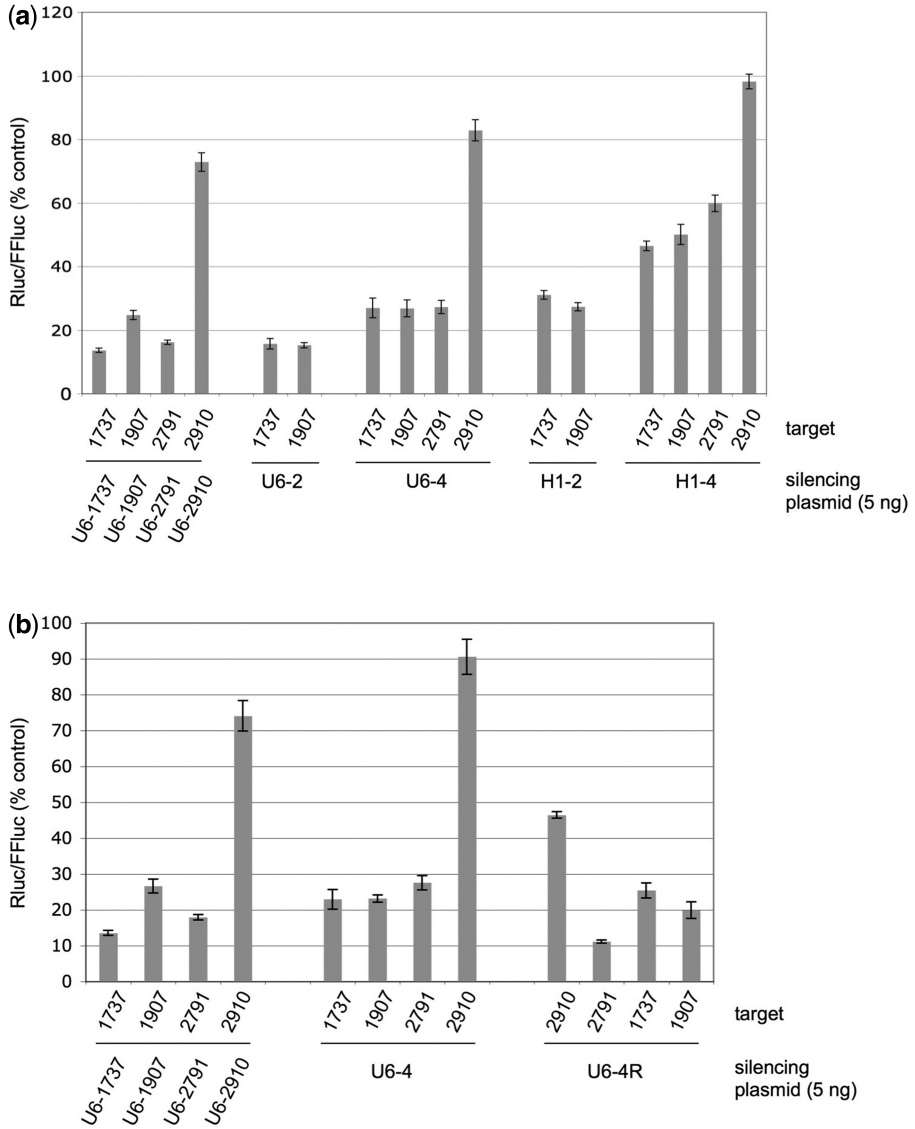

Figure 3. The silencing activity of individual HBV-miRNAs can be affected by their position in the polycistronic transcript. (**a**) Huh7 cells were transfected with 5 ng of monocistronic or polycistronic HBV-miRNA expression plasmids, together with 250 ng of a dual luciferase reporter plasmid carrying target for an individual HBV-miRNA, as indicated. (**b**) Huh7 cells were again cotransfected with monocistronic or polycistronic silencing plasmids together with a reporter plasmid carrying individual target sequences, as indicated. Here, the order of insertion of the HBV-miRNAs has been rearranged in the U6-4R construct relative to U6-4 (Figure 1). Data represent the average (±SD) of triplicate assays of two separate transfections.

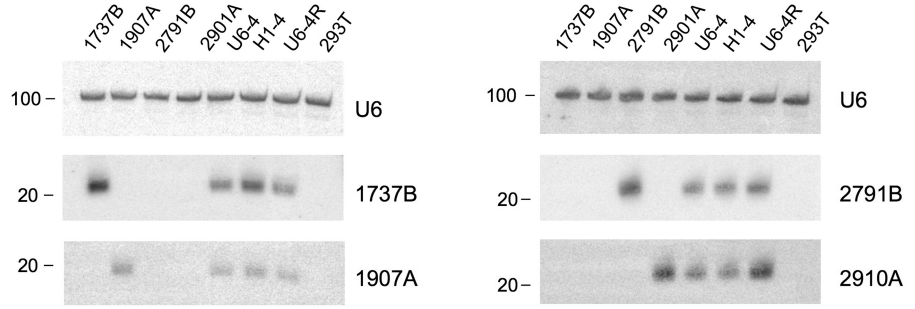

Figure 4. Fully processed miRNA can be detected for each component of the polycistronic HBV-miRNA transcript. RNA was isolated from HEK-293T cells either not transfected or transfected with monocistronic or polycistronic vectors expressing HBV-miRNAs, as indicated above each lane. Two different blots (right and left panels) were probed sequentially for miRNAs or U6 RNA, as indicated to the right of each image.

is 42% lower in these cells when transfected with psiCH-CAT1454, relative to psiCH-CAT360 (Figure 6c). Similar results were observed by Bhattacharyya *et al.* (25), using their reporter plasmids. However, a stronger suppression of Renilla luciferase is observed in Huh7 cells, where Rluc/Fluc activity from psiCH-CAT1454 is reduced by 74% relative to that from psiCH-CAT360, reflecting silencing by miR-122 (25). With the addition of increasing amounts of the HBV-miRNA bicistronic expression vector pUC-U6-2, there was little recovery of Rluc expression in Huh7 cells. Although a slight and apparently dose-dependent increase in the Rluc/Fluc ratio was observed with higher doses of pUC-U6-2, the difference from control values did not reach statistical significance, even at levels that provided strong silencing of HBV targets (Figure 2).

In another set of experiments, we tested whether HBV-miRNAs expressed from our plasmids can compete for processing of a co-expressed miRNA. Here, HEK-293T cells were transfected with a plasmid that expresses a destabilized form of EGFP, pCMV-dsEGFP and the silencing plasmid, pUC-U6-30/EGFP, in the absence or presence of increasing amounts of potential competitor plasmids, pUC-U6-2 or pUC-U6-4. Two days posttransfection, cells were lysed and assayed by immunoblot analysis for the expression EGFP. Results in Figure 6d show that EGFP is effectively silenced by pUC-U6-30/EGFP, and there is little or no suppression of that activity even in the presence of high doses of HBV-miRNA expression plasmids. Therefore, we see minimal evidence

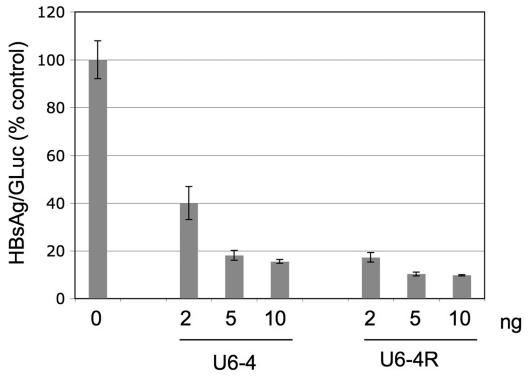

Figure 5. Antiviral activity of the polycistronic HBV-miRNA plasmids. Huh7 cells were transfected with 100 ng pHBV/2, an infectious molecular clone of HBV, together with no silencing plasmid or increasing amounts of pUC-U6-4 or pUC-U6-4R, as indicated. pGLuc (50 ng) was included as a transfection efficiency control. Two days posttransfection, culture supernatants were assayed for HBV S-antigen (HBsAg) and results were normalized to secreted GLuc activity. Results are reported as percent of HBsAg secretion in the absence of silencing plasmid and represent the average (±SD) of two separate transfections assayed in triplicate.

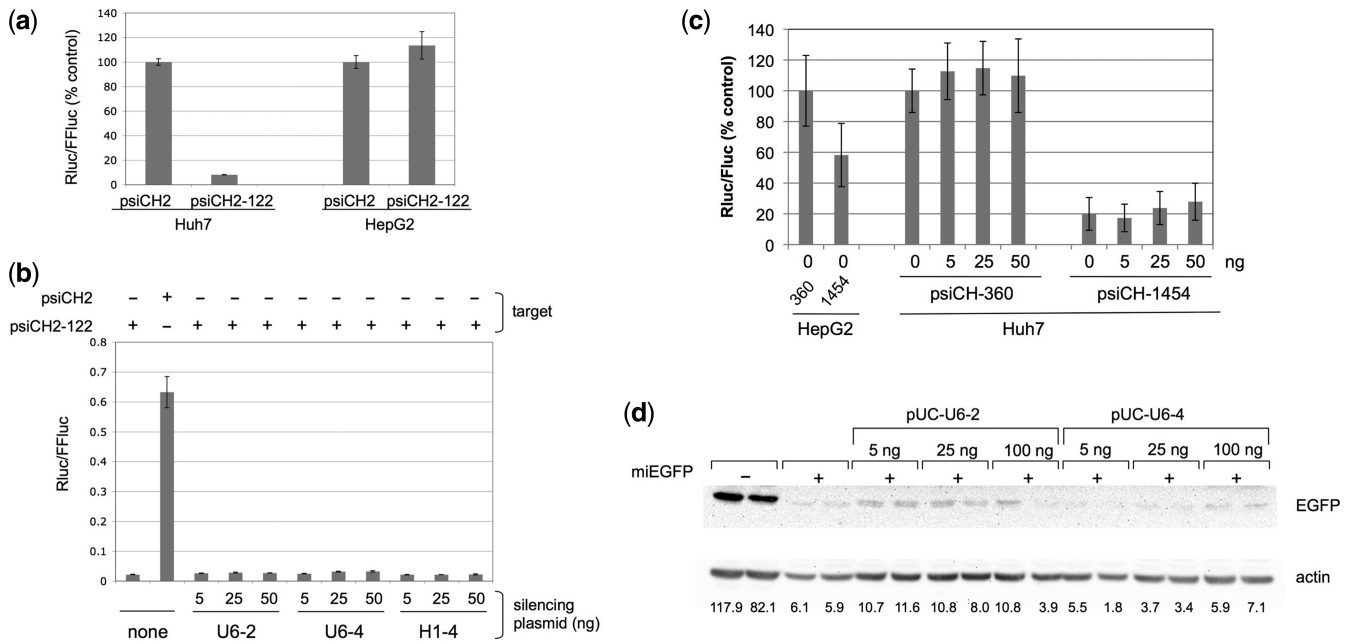

Figure 6. The polycistronic HBV-miRNAs show minimal competition for activity of endogenous miR-122 in Huh7 cells or exogenously expressed miEGFP in HEK-293T cells. (**a**) Huh7 cells or HepG2 cells were transfected with psiCHECK-2 (psiCH2) or with psiCH2-122 where perfectly matched target sequence for miR-122 has been inserted downstream of the Renilla luciferase coding region. Results represent the average (±SD) of two separate transfections assayed in duplicate. (**b**) Huh7 cells were transfected with psiCH2-122 together with no silencing plasmid, or with increasing amounts of pUC-U6-2, pUC-U6-4 or pUC-H1-4 as indicated. As a control for reporter expression, cells were transfected with the psiCH2 plasmid, in the absence of silencing plasmid. Results show the average (±SD) of three separate transfections assayed in triplicate. For both (a) and (b), cells were lysed 2 days posttransfection and luciferase activity was assayed as in Figure 2. (**c**) HepG2 or Huh7 cells were transfected with psiCH-CAT360 or psiCH-CAT1454 together with the indicated amount of pUC-U6-2. Cells were lysed 3 days posttransfection and assayed as in Figure 2. Results show the average (±SD) of three separate transfections for HepG2 cells, and six transfections for Huh7 cells. (**d**) 293T cells were transfected with pCMV-dsEGFP in the absence of silencing plasmid, or with a constant amount of pUC-U6-miEGFP silencing plasmid plus increasing amounts of pUC-U6-2 or pUC-U6-4, as indicated. Immunoblot analysis of protein extracts prepared 2 days posttransfection was carried out using α-EGFP and α-actin antibodies. Duplicate lanes show results from two separate transfections for each set of experimental conditions. Numbers below each lane indicate the percent EGFP present relative to control (average of no miEGFP values) after normalization to actin.

of competition for the activity of an endogenous, largely pre-existing miRNA (miR-122) or for the processing of a co-expressed miRNA (miEGFP) in the presence of the pol III driven polycistronic HBV-miRNAs.

DISCUSSION

In the work presented here, we have shown that it is possible to drive polycistronic expression of miRNAs from a pol III promoter. This is an important addition to the current selection of designs available for the expression of multiplexed silencing RNAs that has the advantage of producing high levels of multiple miRNAs from a compact cassette. The miRNAs are processed from a single precursor and are expressed at similar levels so that overall silencing is not dominated by one member of the set. The expression unit is small and nonrepetitive and can be incorporated into a variety of plasmid or viral vectors. Plasmids designed to express as many as four HBV-miRNAs show strong silencing of viral targets in reporter systems and in a cell culture model of viral replication.

Most endogenous miRNAs are transcribed from pol II promoters (29,30) and several groups, including our own, have used these promoters to construct vectors for multiplexed expression of silencing RNAs designed to resemble miRNAs (14–17,31–33). Pol II promoters allow a degree of targeting by cell type-specific expression or regulation, and therefore can be a good choice for use in vectors that will be delivered broadly to a range of tissues in an organism. However, there can also be advantages to the stronger expression possible from pol III promoters, particularly when using low or single-copy vectors. Until now, the use of pol III promoters has been limited by their ability to express only a single shRNA, making it necessary to introduce a separate promoter to drive each shRNA in vectors designed for multiplexed expression (8–12). The finding that naturally occurring miRNAs can be encoded directly downstream of Alu repeat sequences and processed from these highly structured pol III transcripts (26) suggested that it would be possible to express several functional interfering RNAs from a single pol III promoter if they were formatted as miRNAs. Although the Alu transcripts are driven by type 2 pol III promoters, with elements internal to the transcript, we chose to use U6, H1 and 7SK (not shown) type 3 promoters that are located upstream of the transcription start site and are, therefore, more convenient to use in gene constructions. We show that pol III is capable of transcribing through at least four structured hairpin regions and that functional miRNAs can be processed from these transcripts. We also show that the order in which particular hairpins are encoded in the polycistronic transcript can affect their silencing potency. Whether this is due to sequence elements or structure that might cause premature termination of transcription or to sequence-specific folding or processing effects needs to be studied further.

While pol III promoters are naturally ubiquitously and constitutively expressed, they can be modified and made inducible (34–36). Since their regulation would require the expression of nonendogenous transactivator or repressor proteins, this could be difficult to implement therapeutically. Nevertheless, the use of inducible pol III promoters could be a useful modification to our vector in research applications.

Strong silencing activity, together with multiplexed targeting, has been shown to be important in maximizing efficacy and reducing viral escape both by mathematical modeling (18) and by experimentation (12,13,19). At the same time, however, high levels of shRNA produced from pol III promoters have been associated with cellular toxicity. For instance, AAV vectors directing the expression of certain shRNAs in the liver were lethal to mice when delivered at high dose (20). Similarly, neurotoxicity observed in the brains of mice injected with shRNA-AAV vectors correlated with high levels of shRNA production (21). Additional studies have indicated that expression of shRNAs from a strong U6 promoter in lentiviral vectors can lead to potent silencing of targets in transduced peripheral blood lymphocytes, but also causes cytotoxicity, while lower levels of expression from the H1 promoter are tolerated (19). Although not fully understood, the toxicity caused by high levels of shRNA expression has been attributed to saturation or competition for components of the endogenous miRNA processing pathway (20,22) or to off-target effects caused by the accumulation of excessive amounts of antisense RNAs (21).

Importantly, evidence is emerging that silencing RNAs that are produced in the format of endogenous miRNAs, and presumably undergo nuclear processing by the Drosha/DGCR8 complex, do not display the same toxicities as shRNAs even though they can have comparable or better silencing potency (21,22). In hepatocytes transfected with relatively large amounts of our pol III driven polycistronic HBV-miRNA expression plasmids, we see minimal reductions in the silencing activity of endogenous miR-122 against either a perfectly matched target sequence or against imperfectly matched, confirmed targets from the 3′-UTR of human CAT-1 mRNA. We also see little evidence of competition for miRNA processing when these plasmids are cotransfected into HEK-293T cells with a pol III driven miEGFP expression plasmid. It is important to note, however, that the results of competition as evaluated in transient transfection assays can be influenced by the ratio of target to interfering RNA, the ratio of interfering RNA to competitor, the sequence of the interfering RNA and available processing pathway components in any given cell type (37–39). Undoubtedly there are levels of expression at which these pathways become saturable. While more stringent testing in animal models will be necessary to characterize potential toxicities, our initial results suggest that potent silencing can be achieved from small amounts of vector without triggering the strong competitive effects associated with shRNA expression.

In summary, we describe the use of RNA pol III promoters for polycistronic expression of interfering RNAs. We find that formatting them as miRNAs allows the processing and expression of at least four interfering RNAs from a single pol III transcript, providing potent silencing without the evidence of saturation of endogenous

miRNA pathways. This compact design will add to the versatility of silencing vectors in both research and therapeutic applications.

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