Antiviral Research 80 (2008) 36–44

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Vector design for liver-specific expression of multiple interfering RNAs that target hepatitis B virus transcripts

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ARTICLE INFO

Article history:
Received 11 January 2008
Accepted 10 April 2008

Keywords:
RNAi
Hepatitis B virus
Anti-viral therapy
MicroRNA
RNA pol II promoter
Expression vector

ABSTRACT

RNA interference (RNAi) is a process that can target intracellular RNAs for degradation in a highly sequence-specific manner, making it a powerful tool that is being pursued in both research and therapeutic applications. Hepatitis B virus (HBV) is a serious public health problem in need of better treatment options, and aspects of its life cycle make it an excellent target for RNAi-based therapeutics. We have designed a vector that expresses interfering RNAs that target HBV transcripts, including both viral RNA replicative intermediates and mRNAs encoding viral proteins. Our vector design incorporates many features of endogenous microRNA (miRNA) gene organization that are proving useful for the development of reagents for RNAi. In particular, our vector contains an RNA pol II driven gene cassette that leads to tissue-specific expression and efficient processing of multiple interfering RNAs from a single transcript, without the co-expression of any protein product. This vector shows potent silencing of HBV targets in cell culture models of HBV infection. The vector design will be applicable to silencing of additional cellular or disease-related genes.

1. Introduction

Chronic infection with hepatitis B virus (HBV) is a serious global public health problem in need of improved therapies. Although an effective vaccine is available, more than 350 million people worldwide and 1.25 million people in the U.S. are chronically infected. Long-term infection leads to a high risk of progression to cirrhosis and hepatocellular carcinoma and these diseases are responsible for as many as 1 million deaths each year (Lee, 1997; WHO, 2000).

Currently approved therapies for HBV are interferons, that act as immunomodulators, and nucleoside/nucleotide analogs, that act as viral polymerase inhibitors (Zoulim, 2006; Lok and McMahon, 2007). In general, these therapies suffer from a range of limitations such as incomplete efficacy, poor patient tolerance, lengthy treatment regimens, and the selection of viral escape mutants. While these problems are being addressed, in part, with the continuing development of new nucleoside polymerase inhibitors with improved efficacy and resistance profiles, it is clear that a new and different approach to HBV therapy can make an important contribution to the treatment of this disease.

RNA interference (RNAi) is a process by which gene expression can be silenced in a sequence-specific manner, making it a powerful tool that is being pursued in many therapeutic applications. RNAi-based anti-viral strategies are particularly attractive since infection produces a unique set of viral transcripts that can serve as therapeutic targets. Certain aspects of HBV biology and pathology make it a good candidate for RNAi-based therapies (Lee, 1997; Romano et al., 2006). For example, the virus replicates through an RNA intermediate, so interfering RNAs can directly down-regulate viral replication and the production of infectious viral particles. In addition, the large excess of viral antigens, relative to infectious particles, produced by infected hepatocytes from either episomal or integrated forms of the viral genome, is a significant component of immune response-mediated HBV pathogenesis and persistence (Chisari and Ferrari, 1995). The ability to reduce viral antigen production by silencing viral mRNAs is an important feature of RNAi-based strategies that is not shared by nucleoside analog inhibitors of the HBV polymerase (Romano et al., 2006).

The potential value of RNAi-based approaches in treating HBV infection has been demonstrated in many studies that have used interfering RNAs to down-regulate viral transcripts in both cell culture and animal models of infection (reviewed in (Radhakrishnan et al., 2004)). Interfering RNAs that target HBV have been transfected into cultured hepatocytes either as pre-formed, synthetic, short interfering RNAs (siRNAs) (Hamasaki et al., 2003), or as

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doi:10.1016/j.antiviral.2008.04.001

plasmids that express short hairpin RNAs (shRNAs) (Shlomai and Shaul, 2003; Liu et al., 2004; Zhang et al., 2004; Moore et al., 2005; Romano et al., 2006). In a variety of mouse models of infection, HBV-targeted interfering RNAs have reduced viral antigen, transcript, or DNA production when delivered as siRNAs or as shRNAs expressed from plasmid or viral vectors (Giladi et al., 2003; McCaffrey et al., 2003; Morrissey et al., 2005a,b; Uprichard et al., 2005; Romano et al., 2006; Ying et al., 2007). While many methods have been successful in laboratory studies, it remains a challenge to deliver RNAi agents to hepatocytes in a manner that will be clinically relevant in human patients.

In the current work we describe a new vector designed for efficient processing of multiple interfering RNAs from a single transcript that can be expressed from an RNA polymerase II promoter with the potential for tissue-specific expression. The vector incorporates many features of endogenous microRNA (miRNA) gene organization that are proving useful for the development of reagents for RNAi. We expect that enhanced expression and potency of interfering RNAs in hepatocytes will augment continuing progress in the development of formulations for liver-directed delivery. Further, the expression of multiple interfering RNAs from a single transcript can provide targeting against a range of HBV serotypes and protection from the selection of viral escape mutants.

2. Materials and methods

2.1. Plasmid constructions

Plasmids of the pUC-U6-30/X series are designed for expression of miRNAs from an RNA pol III promoter (U6) and are based on the expression cassette contained in Expression Arrest™ library plasmids (Silva et al., 2005) (Open Biosystems, Huntsville, AL). To facilitate manipulation and growth of the plasmids, the U6 promoter and miR-30 regions from a library plasmid that targets EGFP mRNA were removed from the pSM2 backbone and recloned into pUC19, to make pUC-U6-30/EGFP. The fragment encoding the stem–loop targeting EGFP was removed by digestion with XhoI and EcoRI and replaced with a 110 bp fragment assembled from oligonucleotides and designed to encode a 21 nt stem region targeting HBV RNAs. The sequence of the various HBV-targeted stem–loop regions was based on results using a design algorithm at the Hannon Lab web site (Hannon, 2006) to search conserved regions near positions 1737, 1907, 799, and 2791 in the HBV genome (Romano et al., 2006). At least two alternative HBV-miRNAs, targeting slightly shifted sequence in each region, were tested for each conserved region (see Table 1). Genomic sequence positions refer to HBV strain ayw, GenBank accession number V01460.1.

For plasmids of the pLV-30s/X and pCMV-30s/X series, the stem–loop region plus approximately 30 bp of flanking sequence from pUC-U6-30/X plasmids was copied by PCR, using primers that added XbaI and SpeI sites to the 5′ and 3′ ends, respectively. These fragments were cloned into a unique ClaI site in the second intron of pLIVE (Mirus Bio, Madison, WI) that had been modified by removal of XbaI and SpeI sites present in the vector. Standard techniques were used to destroy the ClaI site and retain the XbaI and SpeI sites surrounding the inserted stem–loop fragment. To construct the bicistronic plasmid pLV-30s/1737B/1907A (pLV-30s/DInt2), the monocistronic 1737B plasmid was digested with SpeI, and an XbaI/SpeI fragment with the 1907A stem–loop was inserted by ligation. The polycistronic plasmid pLV-30s/QInt2 was similarly constructed by the sequential addition, into the SpeI site, of XbaI/SpeI fragments carrying the stem–loop regions targeting the 799A and 2791B HBV regions. The entire cluster of four HBV-miRNAs was removed from pLV-30s/QInt2 by digestion with XbaI and SpeI, blunted, and inserted into the SmaI site in the exonic MCS of modified pLIVE to generate pLV-30s/QEx, or into the ScaI site (blunted) in intron 1 of modified pLIVE to generate pLV-30s/QInt1. The liver-specific promoter of pLIVE was removed by digestion with BglII and KpnI and replaced with the CMV-IE promoter to make pCMV-30s/X plasmids. All constructs were confirmed by DNA sequencing.

Dual luciferase reporter plasmids containing HBV target sequence were constructed in psiCHECK-2 (Promega, Madison, WI). psiCH-HBV21/20 contains HBV sequence from positions 2901 to 1696 in the HBV genome with targets for both 1737 and 1907 miRNAs; psiCH-HBV-1737 contains HBV sequence from 1802 to 1696 with targets for 1737 miRNAs; psiCH-HBV-1907 contains HBV sequence from 1963 to 1853 with targets for 1907 miRNAs; psiCH-HBV-799 contains HBV sequence from 907 to 675 with targets for 799 miRNAs; psiCH-HBV-2791 contains HBV sequence from 2932 to 2671 with targets for 2791 miRNAs; psiCH-HBV contains the entire HBV genome, linearized at the unique EcoRI site and inserted immediately downstream of the 799 target region in psiCH-HBV-799, so that all relevant HBV targets are present in the 3′UTR of the transcript. All plasmids were made by PCR amplification of the appropriate genomic region and subsequent ligation into the XhoI site of psiCHECK-2. Constructions were confirmed by DNA sequence analysis.

2.2. Cell culture

Human hepatoblastoma Huh7 cells were grown in RPMI 1640 supplemented with 10% FBS. HeLa cells were grown in DMEM supplemented with 4.5 g/L glucose, L-glutamine, and sodium pyruvate, 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.3. HBV silencing assays

Silencing activity of pUC-U6-30/X plasmids was assayed by transfection of Huh7 cells. Cells were plated in 6-well plates and transfected using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells in each well were transfected with 100 ng pM1-SEAP (Roche Applied Science, Indianapolis, IN) as a transfection efficiency control, 500 ng pHBV/2 (HBV strain ayw infectious molecular clone, containing two head-to-tail copies of the HBV genome linearized at the EcoRI site, kindly provided by Dr. F. Chisari), and increasing amounts of silencing plasmid, ranging from 0 to 200 ng. The total amount of DNA in each transfection was kept constant at 2.5 μg by the addition of empty vector DNA. 48 h after transfection, culture supernatants were assayed for SEAP activity using a SEAP Reporter Gene Assay Kit (Roche) and for HBV surface antigen (HBsAg) using Genetic Systems HBsAg EIA 3.0 (Bio-Rad Laboratories, Hercules, CA). HBsAg levels were normalized to SEAP activity and results reported as percent of normalized HBsAg in control wells where no silencing plasmid was added. Silencing activity of pLV-30s/QInt2 was also tested against

Table 1
HBV (strain ayw) genomic sequence corresponding to targeted regions of HBV transcripts

1737B: GACGTCCTTTGTTTACGTCCCGTCG**GCGCTGAATCCTGCGGACGAC**CCTTCTCG
1737C: GACGTCCTTTGT**TTACGTCCCGTCGGCGCTGAA**TCCTGCGGACGACCCTTCTCG
1907A: TGGAACCTTT**TCGGCTCCTCTGCCGATCCAT**ACTGCGGAACTCCTAGCCGCTTG
1907B: TGGAACCTTTTCGGCTCCTCTGCCG**ATCCATACTGCGGAACTCCTA**GCCGCTTG
799A: GAAGAAGAAC**TCCCTCGCCTCGCAGACGAAG**GTCTCAATCGCCGCGTCGCAGAA
799B: GAAGAAGAACTCCCTCGCC**TCGCAGACGAAGGTCTCAATC**GCCGCGTCGCAGAA
2791A: ACTTGTCCT**GGTTATCGCTGGATGTGTCTG**CGGCGTTTTATCATCTTCCTCTTC
2791B: ACTTGTCCTGGTTATC**GCTGGATGTGTCTGCGGCGTT**TTATCATCTTCCTCTTC

the pHBV/2 infectious molecular clone. Huh7 cells in 6-well plates were co-transfected with pHBV/2 and pLV-30s/QInt2 using Arrest-In (Open Biosystems). A constant amount of pGLuc (New England Biolabs, Beverly, MA) was included as a transfection efficiency control. Two days post-transfection, secreted HBsAg was measured, as above, and values were normalized to secreted luciferase activity, measured using the Gaussia Luciferase Assay Kit (New England Biolabs).

In many cases, silencing was measured using constructs in the dual luciferase reporter plasmid, psiCHECK-2, described in Section 2.1. Arrest-In was used to transfect Huh7 cells with the reporter plasmid and silencing plasmids as indicated in figure legends. The total amount of DNA used in transfections was kept constant by the addition of empty vector. Two days post-transfection, cells were lysed and firefly and Renilla luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega).

HeLa cells were transfected using FuGene (Roche) according to manufacturer's instructions. The cells were plated in 96-well plates and transfected with 25 ng of the reporter plasmid psiCH-HBV21/20 and 1, 5, and 10 ng of pLV-30s/DInt2 or pCMV-30s/DInt2. Total DNA was kept constant by the addition of empty vector. One day post-transfection the cells were lysed and the luciferase activities were assayed, as above.

The quantity of plasmid used in transfections has been converted to pM amounts in the figures to allow comparisons between transfections using different plate formats.

2.4. Quantitative RT-PCR assays

Total RNA isolated from transfected cells was analyzed using TaqMan Gene Expression assays according to manufacturer's directions. Values for each mRNA (p56, IFN-β, and MX-1) were normalized to 18s RNA and expressed relative to levels found in untreated cells.

2.5. Northern blot analysis of miRNA expression and processing

Huh7 cells were plated in 6-well plates and transfected with 2 μg of HBV-miRNA expression plasmid using Arrest-In (Open Biosystems) according to manufacturer's instructions. 48 h post-transfection, cells were collected and total RNA was isolated using a mirVana RNA isolation kit (Ambion, Austin, TX). For each sample, 10 μg of RNA was separated by electrophoresis in a 12% polyacrylamide urea–TBE gel and then electro-blotted to BrightStar Plus nylon membrane (Ambion). Blots were vacuum dried at 60 °C for 1 h, pre-hybridized at 41 °C for 1 h in ULTRAhyb-Oligo hybridization buffer (Ambion), and then ^{32}P-radiolabeled oligonucleotide probe was added for overnight hybridization at 41 °C. Probes were 21 nt oligonucleotides complementary to the anti-sense (guide) strand of mature HBV-miRNAs. Hybridization was detected using a GE Healthcare Storm 820 phosphorimager.

3. Results

3.1. Efficacy of HBV-targeted interfering RNAs formatted as miRNAs

Previous work has identified regions in the HBV genome that are well-conserved among HBV serotypes and susceptible to silencing by expressed interfering RNAs (Romano et al., 2006). These regions are found throughout the 3.2 kb genome and, because of the highly overlapping pattern of transcription, can include sequences that are simultaneously present on the 3.5 kb pregenomic RNA and one or more of the mRNAs that encode viral proteins, as depicted in Fig. 1. A plasmid vector has been generated for therapeutic applications, where four different expression cassettes, each using an RNA pol III promoter to drive transcription of an shRNA, have been combined in a single plasmid that provides potent silencing of HBV transcripts in cell culture assays and mouse models of HBV infection (Romano et al., 2006). In the design of a second-generation of vectors described here, we have used an miRNA format in order to capture the advantages of using an RNA pol II promoter, with the potential for tissue-specific expression, to drive expression of multiple interfering RNAs from a single transcript. In our design, it is not necessary for the vector to co-express any protein product.

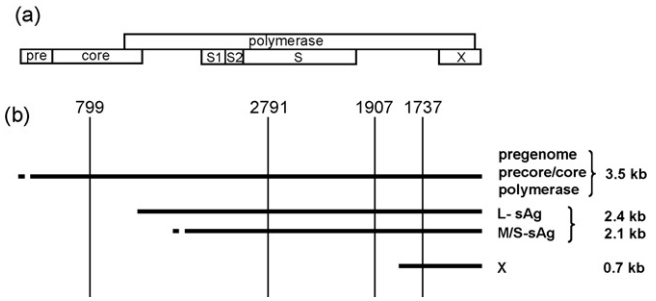

Fig. 1. Four major transcripts from the HBV genome are 3′-coterminal and encode both the HBV pregenome and HBV proteins. (a) Schematic representation of overlapping open reading frames (ORFs) that encode the HBV proteins. (b) HBV transcripts are aligned with the ORF map, above. The protein products and size, in kilobases, of each transcript are indicated to the right. Vertical lines show the location of four conserved regions that can be present in multiple transcripts. Numbers refer to the position of the conserved regions in the HBV genome.

Initially, we tested individual HBV-targeted interfering RNAs expressed from pol III promoters, but formatted as miRNAs instead of the previously tested shRNAs (see Fig. 2a). We used an expression cassette based on that found in Expression Arrest™ plasmids (Open Biosystems) where a U6 promoter drives transcription of interfering RNAs built into the context of human miR-30 (Silva et al., 2005). We substantially simplified these plasmids by moving the expression cassette out of the pSM2 vector backbone of Expression Arrest™ plasmids and into the plasmid pUC19, generating the pUC-U6-30/X series of plasmids (where X will designate the target). The stem–loop region was reconfigured to target conserved regions found in HBV transcripts, using an si/miRNA design algorithm. For the conserved regions surrounding HBV genomic positions 1737 and 1907 (see Fig. 1), we tested two alternative miRNAs that differ only slightly in the position of their target sequence (see Table 1). The efficacy of each HBV-miRNA was tested by assaying hepatitis S antigen (HBsAg) secretion from Huh7 cells after co-transfection with a constant amount of the infectious plasmid pHBV/2 and increasing amounts of silencing plasmid. A SEAP reporter plasmid, pM1-SEAP, was included to control for transfection efficiency. We found that reductions of as much as 90% of HBsAg levels can be achieved even at intermediate doses of the miRNA-formatted silencing plasmids. Representative data for plasmids targeting HBV regions 1737 and 1907 are shown in Fig. 2b. As expected, the silencing response is both dose- and sequence-dependent.

3.2. Expression of HBV-miRNAs from RNA pol II promoters

A potential advantage to formatting interfering RNAs as miRNAs is that they can be expressed from RNA pol II promoters and therefore can be designed to be expressed in a tissue-specific or regulated manner. In our first attempts to introduce a pol II promoter into the pUC-U6-30/X plasmids, we exchanged the U6 promoter for a liver-specific promoter derived from the plasmid pLIVE. In addition, we inserted a BGH 3′UTR downstream of the miR-30 3′

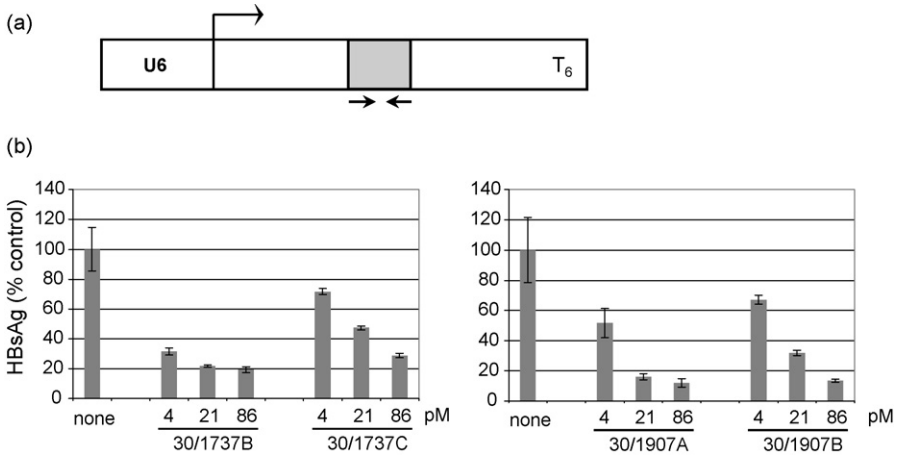

Fig. 2. Silencing activity of plasmids that express miR-formatted interfering RNAs that target HBV RNAs. (a) General structure of the U6 RNA pol III expression cassette for miR-30 formatted interfering RNAs in pUC-U6-30/X plasmids. Approximately 120 nt of sequence on each side of the stem structure and 19 nt of loop sequence are derived from miR-30 (open boxes). The stem–loop region (shaded box with opposing arrows) is designed such that the mature miRNA has complete complementarity to the target RNA. The U6 promoter and the pol III transcription termination site (T_6) are indicated. (b) Silencing activity from pUC-U6-30/X plasmids that target regions near sites at 1737 and 1907 (see Table 1). Huh7 cells were transfected with constant amounts of pHBV/2 (84 pM, equal to 500 ng in 1 mL) and pM1-SEAP together with increasing amounts of the indicated silencing plasmid. The amounts are shown as pM and correspond to 10, 50, and 100 ng used in a 1 mL transfection. Culture supernatant was collected 48 h post-transfection and assayed for SEAP activity and HBsAg. Results are calculated as HBsAg in the culture supernatant, normalized to SEAP activity and expressed as a percent of controls where no silencing plasmid was added. HBsAg values are the average of two assays each for two independent transfections, ±S.D.

flanking sequence to prevent transcriptional readthrough into plasmid sequence. These constructs were inactive in silencing (data not shown). In order to correct this, we generated plasmids of the pLV-30s/X series, where the miRNA stem–loops are inserted into a longer non-protein coding sequence, more closely mimicking natural miRNAs. For these plasmids, we moved the silencing stem–loop of pUC-U6-30/X plasmids into the second intron of the pLIVE vector so that the HBV-miRNA would be processed from a transcript that consists primarily of two introns and contains no open reading frame for the production of a protein (see Fig. 3a). For some constructs, we also exchanged the liver-specific promoter of the pLV-30s/X plasmids for a CMV-IE promoter to create the pCMV-30s/X series of plasmids.

Silencing activity of these pol II driven miRNAs was tested in Huh7 cells co-transfected with the silencing plasmid and a dual luciferase reporter plasmid. HBV target sequence was inserted into the 3′UTR of the Renilla luciferase gene cassette in the vector psiCHECK-2, so that successful silencing is measured as a decrease in Renilla luciferase activity. Expression of firefly luciferase from the same plasmid serves as a transfection efficiency control, and results are determined as the ratio of Renilla to firefly luciferase activity. Results shown in Fig. 3b demonstrate that expression of 1907A HBV-miRNA from the liver promoter (pLV-30s/1907A) is only slightly less effective in silencing than when it is expressed from a U6 promoter (pUC-U6-30/1907A). Use of the stronger CMV pol II promoter (pCMV-30s/1907A) increases the potency of silencing. Importantly, potency was also increased when a second stem–loop that targets the 1737B region was added, generating the plasmid pLV-30s/1737B/1907A (or pLV-30s/DInt2, for double in intron 2). These data suggest that both HBV-miRNAs encoded in the bicistronic plasmid are functional and contribute to silencing of the target RNA.

3.3. Tissue-specific silencing from the RNA pol II promoter

In using a pol II promoter, we expect to be able to select promoters that will enhance the relative expression of therapeutic miRNAs in targeted tissues, even when targeted delivery is not possible. For HBV therapeutic miRNAs it will be advantageous to maximize expression in hepatocytes and minimize expression in

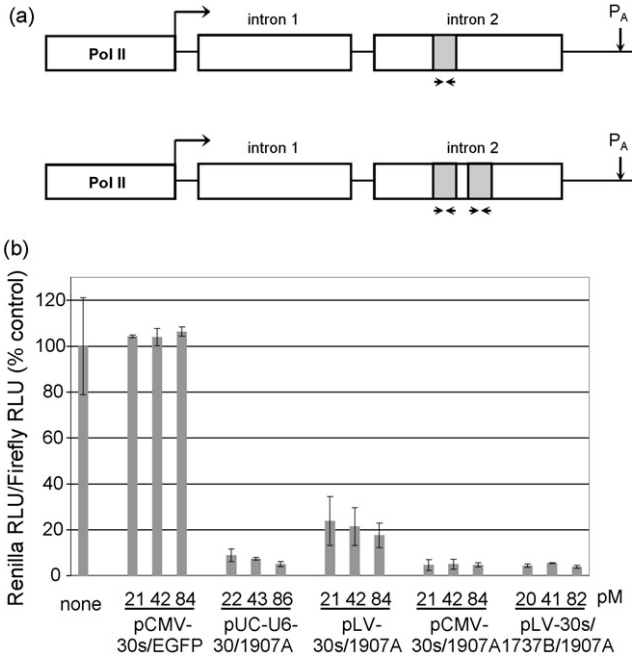

Fig. 3. Silencing activity of miRNAs expressed from RNA pol II promoters. (a) General structure of the pol II expression cassette. A pol II promoter (either the CMV-IE promoter or the liver specific promoter of pLIVE) drives the expression of a transcript that contains two introns (open boxes) and no ORF. An miRNA stem–loop region with ~30 bp of flanking sequence, copied from pUC-U6-30/X plasmids (shaded box with opposing arrows), is inserted into the second intron. One (top) or more (bottom) stem–loops can be inserted into the intron. The start site of transcription (bent arrow) and the polyadenylation site (vertical arrow) are indicated. (b) Silencing activity from RNA pol II driven HBV-miRNAs. Huh7 cells were transfected with a constant amount of psiCH2-HBV21/20 (101 pM, 250 ng in 0.5 mL) together with increasing doses of the indicated silencing plasmid. Amounts are shown as pM and correspond to 25, 50, and 100 ng of silencing plasmid in a 0.5 mL transfection. Two days post-transfection, cells were lysed and assayed for Renilla and firefly luciferase activities. Results are expressed as the ratio of Renilla to firefly luciferase activity, normalized to results from cells with no added silencing plasmid ('none'). Each value represents the average of two assays each for two independent transfections, ±S.D.

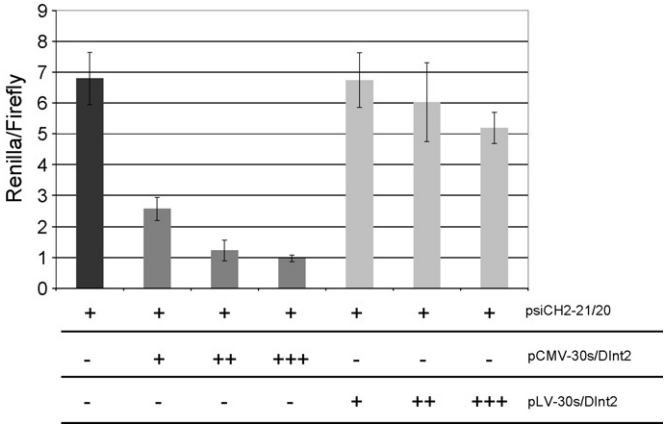

Fig. 4. Tissue specificity of silencing is determined by the pol II promoter. HeLa cells were transfected with the psiCH2-HBV21/20 reporter plasmid (58 pM) together with increasing amounts of pLV-30s/DInt2 or pCMV-30s/DInt2 (4, 20, and 40 pM), as indicated. Two days post-transfection, cells were assayed for Renilla and firefly luciferase activity, as in Fig. 3. Each value represents the average of two assays for four independent transfections, ±S.D.

other tissues, thereby reducing concerns about potentially deleterious effects in non-targeted tissues. The liver-specific promoter from pLIVE, used in our pLV-30s/X plasmids, combines a mouse alpha-fetoprotein enhancer and minimal albumin promoter. To test the tissue specificity of silencing with this promoter, we compared silencing in HeLa cells transfected with the bicistronic plasmid carrying the liver-specific promoter (pLV-30s/DInt2) to that with the broadly active CMV promoter (pCMV-30s/DInt2). Fig. 4 shows that the HBV-miRNAs are capable of strong silencing of an HBV reporter plasmid in these cells, when expressed from a CMV promoter. However, very little silencing was achieved with plasmids using the liver-specific promoter in HeLa cells, most likely due to low levels of expression of the miRNAs. These results demonstrate that cell type directed expression of the HBV-miRNAs is possible, using a pol II promoter.

3.4. Expression of miRNAs does not induce an interferon response

The exogenous expression of interfering RNAs in cells, whether formatted as sh- or miRNAs, leads to the production of highly structured RNAs that have the potential to induce an interferon response. To test whether the miRNAs produced from our bicistronic plasmid trigger an interferon response, we transfected cells and assayed several mRNAs that serve as markers for this response. In these experiments, we used HeLa cells since they are capable of a more robust interferon response than Huh7 cells. The plasmid pCMV-30s/DInt2 was transfected into cells in an amount higher than required for >85% reduction in HBV reporter activity (see Fig. 3). Quantitative RT-PCR was used to determine levels of mRNA encoding p56 (IFIT-1), IFN-β, and MX-1 in transfected cells relative to levels in untreated HeLa cells. As shown in Fig. 5, very little induction of any of these markers was observed at either 6 h or 24 h post-transfection, as compared to the response induced by transfection with poly(I:C), used as a positive control. In fact, even the minimal induction of p56, the most sensitive of the markers tested, appears to have been caused by introduction of the vector itself, not by expression of the miRNAs. After transfection with the empty vector, pCMV-LV, that produces no miRNA, the low level of p56 induction is comparable to that seen when pCMV-30s/DInt2 is transfected (see Fig. 5a). We conclude that, at levels above those necessary for efficacy in silencing HBV targets, there is no significant induction of an interferon

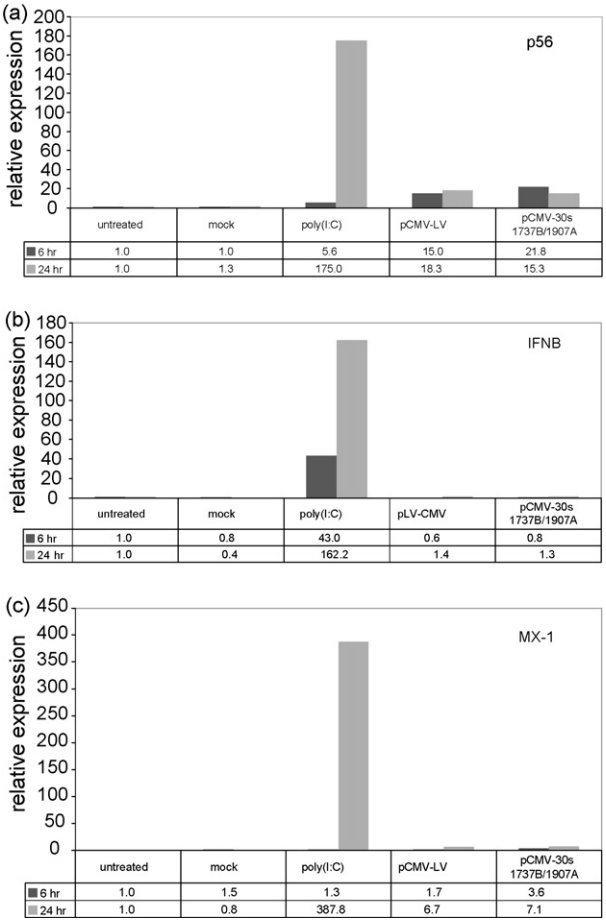

Fig. 5. Expression of miRNAs does not induce an interferon response. Total RNA was isolated from HeLa cells 6 h and 24 h after transfection with 2 μg/mL poly(I:C), mock transfection, transfection with pCMV-LV (no miRNA), or transfection with pCMV-30s/1737B/1907A (pCMV-30s/DInt2). Silencing plasmids were used at 51 pM. Quantitative RT-PCR was used to measure levels of (a) p56 mRNA, (b) IFN-β mRNA, and (c) MX-1 mRNA. Results are presented as expression relative to levels found in untreated HeLa cells and represent the average of three reactions.

response by the exogenous expression of the HBV-targeted miRNAs.

3.5. Polycistronic plasmid expressing four HBV-miRNAs from a single RNA pol II transcript

A goal of this work is to create a plasmid capable of expressing multiple miRNAs targeting different regions of HBV transcripts in order maximize potency while retaining efficacy against a range of viral serotypes and minimizing the selection of escape mutants. Therefore, we included additional HBV-miRNAs in our RNA pol II driven plasmids. Stem–loop structures targeting transcripts from additional conserved regions (HBV genome positions 799 and 2791, see Table 1 and Fig. 1) were designed and tested as monocistronic plasmids in the pUC-U6-30/X format, as described in Section 3.1 (data not shown). The stem–loops targeting regions 799A and 2791B (Table 1) showed the strongest silencing and were added into the bicistronic vector (pLV-30s/DInt2) to generate a plasmid encoding four different HBV-miRNAs (pLV-30s/1737B/1987A/799A/2791B). This polycistronic plasmid is referred to as pLV-30s/QInt2 (for quadruple, in intron 2). This plasmid, schematically depicted in Fig. 6a, was tested for silencing activity using a dual luciferase reporter plasmid containing the entire HBV genomic sequence in the 3′UTR of the Renilla luciferase

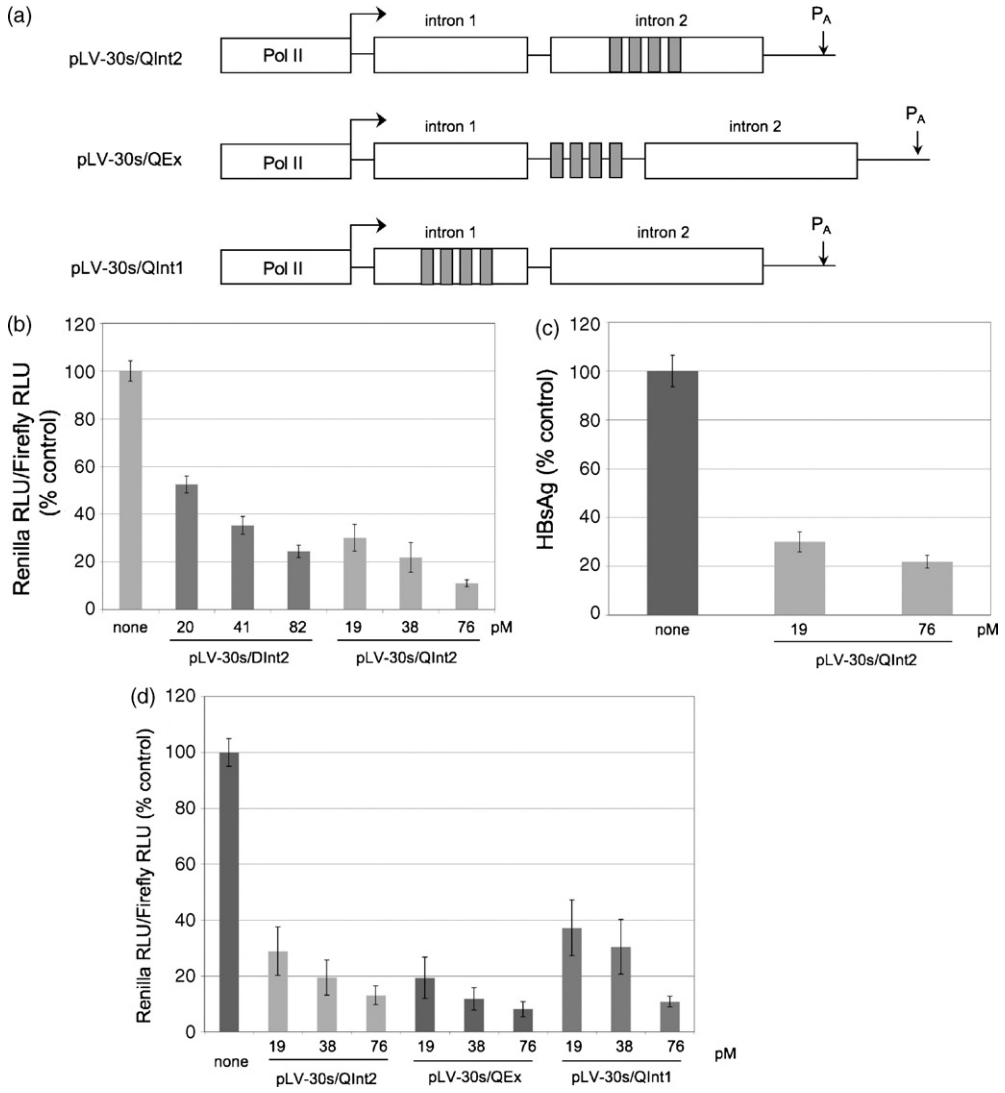

Fig. 6. Silencing potency with increased number and changed location of HBV-miRNAs within the transcipt. (a) Structure of the RNA pol II expression cassettes with four HBV-miRNAs encoded within intron 2 (pLV-30s/QInt2), exon sequence (pLV-30s/QEx), or intron 1 (pLV-30s/QInt1). As tested here, the plasmids use the liver-specific promoter of the pLIVE vector. (b) The silencing activity of pLV-30s/DInt2 was compared to that of pLV-30s/QInt2 in co-transfection assays using the dual luciferase reporter, psiCH-HBV. Results show the average of four transfections, ±S.D. (c) Silencing activity of pLV-30s/Qint2 was tested against the infectious molecular clone, pHBV/2. Huh7 cells were transfected with constant amounts of pHBV/2 (34 pM, equal to 200 ng in 1 mL) two different amounts of pLV-30s/QInt2 (19 pM and 76 pM, corresponding to 50 ng and 200 ng in a 1 mL transfection). pGluc was included as a transfection efficiency control. Culture supernatant was collected 48 h post-transfection and assayed for Gaussia luciferase activity and HBsAg. Results are calculated as HBsAg in the culture supernatant, normalized to Gluc activity and expressed as a percent of controls where no silencing plasmid was added. HBsAg values are the average of three assays each for two transfections, ±S.D. (d) Silencing activity was compared among plasmids where sequence encoding a cluster of four HBV-miRNAs was inserted into different regions of the expression cassette, as depicted in panel a. The psiCH-HBV reporter plasmid was used in co-transfection assays with the silencing plasmids, as in panel b. Results show the average of six transfections, ±S.D.

target gene. As shown in Fig. 6b, the two additional HBV-miRNA stem–loops in the plasmid pLV-30s/QInt2 increased its silencing potency by approximately two-fold relative to pLV-30s/DInt2.

The polycistronic plasmid pLV-30s/QInt2 was also tested against the infectious molecular clone, pHBV/2. Huh7 cells were co-transfected with pHBV/2 and two different doses of pLV-30s/QInt2, and silencing was measured as a decrease in HBsAg secreted into the culture supernatant. The results, shown in Fig. 6c, indicate that the polycistronic silencing plasmid is also active against viral RNAs in the context of transcription from the viral genome and assembly of infectious viral particles.

Many endogenously encoded human miRNAs are found in introns (Rodriguez et al., 2006) and it has been reported that experimentally designed miRNAs can show better silencing activity if inserted into an intron region of the expression cassette (Zhou et al., 2005; Chung et al., 2006). Therefore, we had initially placed miRNA encoding sequence into the second intron of the pLIVE vector. In order to test the importance of the sequence context surrounding the HBV-miRNA cluster in our plasmid, we constructed two additional plasmids (see Fig. 6a). In one of them, pLV-30s/QEx, we placed the miRNA cluster into the multiple cloning site that is between the two introns encoded in pLIVE, generating a plasmid that expresses the miRNAs from the exon of a non-protein coding transcript. In another plasmid, pLV-30s/QInt1, we placed the miRNA cluster into the first intron of pLIVE, generating a plasmid similar to pLV-30s/QInt2, but with the miRNA cluster embedded within a different intron sequence. Silencing activity of these plasmids was tested using a dual luciferase–HBV reporter plasmid. The results, shown in Fig. 6d, indicate that at the highest concentrations, pLV-30s/QInt2, pLV-30s/QEx, and pLV-30s/QInt1 plasmids silenced reporter activity to 13%, 8%, and 11% of control levels, respectively. While we have not confirmed the splicing pattern of transcripts

claimed for the pLIVE vector (Mirus Corp.), intron 1 of this vector (299 bp derived from the first intron of the human factor IX gene) is flanked by 7 bp of authentic splice junction sequence and intron 2 (342 bp derived from intron 14 of the human serum albumin gene) is flanked by 26 bp (5′) and 477 bp (3′) of authentic splice junction sequence, and we anticipate that splicing is correct. Nevertheless, it is evident that the sequence context surrounding the cluster of miRNAs does not cause major changes in their potency.

3.6. Silencing activity and processing of individual miRNAs expressed from a polycistronic plasmid

While the inclusion of additional HBV-miRNA stem–loops improved silencing of HBV targets, we sought direct evidence that each of the stem–loops in our polycistronic plasmid is active. Four different dual luciferase reporter plasmids were constructed that contain HBV target sequence for the 1737, 1907, 799, and 2791 HBV-miRNAs, individually. Each of these reporter plasmids was then used to test silencing by its corresponding HBV-miRNA when expressed either from polycistronic or monocistronic plasmids. In these transfections, we used a level of silencing plasmid that was sub-optimal to ensure that the system was not saturated and differences in activity could be detected. As shown in Fig. 7, each of the reporter plasmids can be silenced by co-transfection with the polycistronic plasmids, pLV-30s/QInt2 and pLV-30s/QEx, to the same extent as they are by the corresponding monocistronic plasmid. We note that little silencing activity is seen from the HBV(799)-miRNA in any format at the dose tested. Although our initial results in testing this 799A stem–loop at higher doses were more promising (data not shown), we have consistently found the 799 region to be difficult to target. However, it is evident that expression from a polycistronic cluster does not reduce the potential silencing activity of the HBV-miRNAs.

In addition to the functional tests presented above, we have examined the expression and processing of the HBV-miRNAs from the polycistronic plasmid in northern blots. Huh7 cells were transfected either with the monocistronic plasmids pLV-30s/1737B, pLV-30s/1907A, pLV-30s/799A, pLV-30s/2791B, or pLV-30s/EGFP, or with the polycistronic plasmids pLV-30s/QInt2 or pLV-30s/Ex. RNA isolated from these cells was analyzed by northern blotting, with sequential detection on blots using oligonucleotide probes for

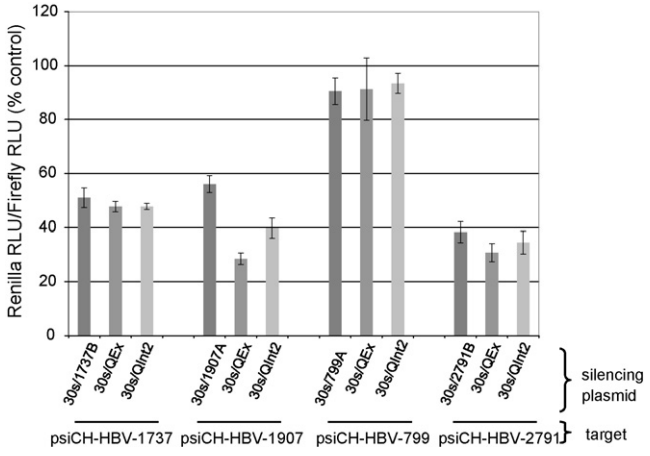

Fig. 7. Functional activity from the individual HBV-miRNAs encoded in a polycistronic silencing plasmid. Silencing activity of the polycistronic plasmids, pLV-30s/QEx and pLV-30s/QInt2, was compared to that of a monocistronic plasmid when tested against a dual luciferase reporter containing the corresponding single HBV target region. Huh7 cells were co-transfected with the indicated silencing plasmid (25 pM pLV-30s/QEx, 25 pM pLV-30s/QInt2, or 28 pM monocistronic plasmid) and target plasmid (119 pM). Two days post-transfection, cells were lysed and assayed for Renilla and firefly luciferase activities. Each value represents the average of three assays for two independent transfections, ±S.D.

the anti-sense (guide) strand of each of the miRNAs. It is evident from the results shown in Fig. 8 that mature miRNAs are expressed and processed at similar levels from mono- and poly-cistronic plasmids. It is noteworthy that the processed HBV(799)-miRNA is also detected, supporting the idea that the apparent low functionality of this miRNA is not due to poor expression or processing. We conclude that it is possible to achieve efficient expression and processing of at least four different miRNA-formatted interfering RNAs from a single, pol II driven, non-protein-coding transcript.

4. Discussion

RNAi is an endogenous cell process that can suppress gene expression in a highly specific manner, and it is providing new approaches for the development of therapies for viral infections

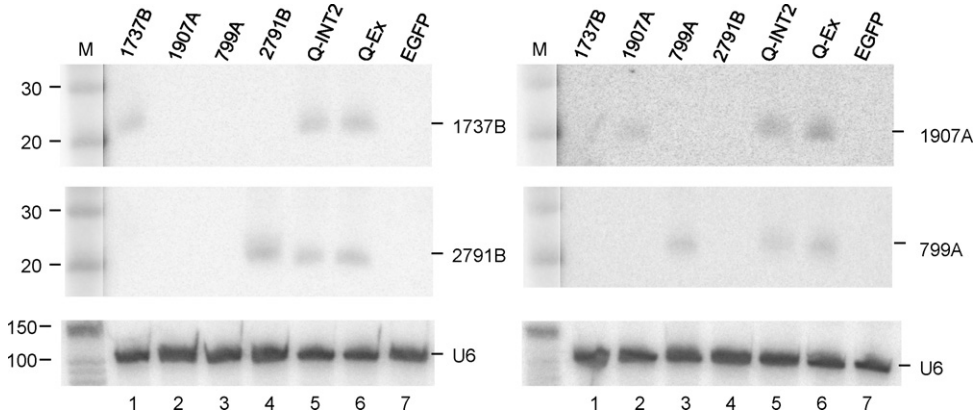

Fig. 8. Mature interfering RNAs are efficiently processed from all cistrons of a polycistronic expression plasmid. RNA was isolated from Huh7 cells transfected with miRNA expression plasmids as follows: lane (1) pLV-30s/1737B; lane (2) pLV-30s/1907A; lane (3) pLV-30s/799A; lane (4) pLV-30s/2791B; lane (5) pLV-30s/QInt2; lane (6) pLV-30s/QEx; lane (7) pLV-30s/EGFP. Lane M contains radiolabeled RNA markers, with sizes indicated in nt. Radiolabeled oligonucleotides were used to probe the blot in the left panels, sequentially, for the anti-sense strand of 1737B, the anti-sense strand of 2791B, and U6 RNA (loading control). Similarly, the blot in the right panels was probed for the anti-sense strand of 1907A, the anti-sense strand of 799A, and U6 RNA. RNA from cells transfected with pLV-30s/EGFP serves as an additional control for hybridization specificity.

and other diseases (Dykxhoorn and Lieberman, 2005). Therapeutic interfering RNA can be directly introduced into cells as exogenously produced siRNA or can be expressed within cells from vector-based systems. Silencing mediated by synthetic siRNAs has been shown to be highly effective (Caplen et al., 2001; Elbashir et al., 2001), although it can be short-lived in *in vivo* applications (Giladi et al., 2003). Recent progress in chemical modification of siRNAs has improved both the longevity and specificity of these agents (Morrissey et al., 2005a,b; Jackson et al., 2006; Zimmermann et al., 2006). Nevertheless, plasmid or virus-based vectors designed for cellular expression of interfering RNAs may provide longer lasting effects and may therefore be most appropriate for the treatment of chronic diseases. We are pursuing strategies for expression of interfering RNAs for the treatment of HBV and other viruses. In this work, we have designed a vector with an RNA pol II driven gene cassette that leads to efficient expression and processing of multiple, user-designed miRNAs from a single transcript, without the co-expression of any protein product. This vector shows potent silencing of HBV targets.

In early work on vector-based RNA silencing, pol III promoters were chosen to drive the expression of shRNAs that can be processed by intracellular enzymes into mature interfering RNAs (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Yu et al., 2002). The strong and ubiquitously active pol III promoters, with defined start and stop sequences, are well-suited for transcription of these short RNAs. Since then, however, as an understanding of the organization, expression, and processing of miRNAs has grown, these endogenously encoded RNAs have provided another model for vector-based expression of interfering RNAs. Of particular importance was the finding that when an endogenous miRNA is redesigned so that it has full complementarity to a target of choice, it can mediate the degradation of the target RNA; and, silencing activity is retained if the stem–loop region, plus some flanking sequence, is placed into the context of an irrelevant RNA (McManus et al., 2002; Zeng et al., 2002; Boden et al., 2004). Further, it was found that miRNAs are often encoded in clusters where multiple miRNAs are processed from a single transcript (Lagos-Quintana et al., 2001; Lee et al., 2002; Altuvia et al., 2005) and that transcription is naturally driven by pol II promoters (Cai et al., 2004; Lee et al., 2004).

In previous work, a therapeutic silencing vector that targets HBV with four separate pol III driven shRNA cassettes showed efficacy in cell culture and animals models of infection (Romano et al., 2006). In the second-generation vector system described here, we have incorporated several aspects of RNA pol II miRNA-formatted expression to take advantage of its potential for polycistronic and tissue-specific expression. Work by others had demonstrated that silencing from miRNAs embedded in RNA pol II transcripts was most effective when no poly(A) site was inserted into the vector (allowing transcriptional readthough into downstream vector sequences) or when protein coding sequence was included in the transcript (Dickins et al., 2005; Silva et al., 2005; Stegmeier et al., 2005). While co-expression of a reporter protein can have value in experimental systems (Dickins et al., 2005; Zhou et al., 2005) it is not useful in a clinical application where the co-expressed protein could be antigenic or toxic. Therefore, we sought to correct this by inserting the HBV-miRNA constructs into a longer transcript, but one that does not encode a protein.

Since endogenous miRNAs are often processed from introns found in mRNAs, we initially inserted the HBV-targeted miRNA sequence into an intron in the liver-specific expression plasmid, pLIVE. Because we did not add any protein coding sequence, transcripts from this construct should consist almost entirely of two introns, with one containing the inserted miRNA cluster. We also tested the effects of positioning the miRNA cluster in putative exonic sequence or in a different intron in the same vector. We did not observe a strong advantage to locating the miRNA cluster within an intron. Instead, we have seen a small but reproducible increase in silencing when the miRNA cluster is placed in an exon, in the pLIVE vector background as well as in totally unrelated vectors (data not shown).

The ability to express multiple miRNAs is important to our overall strategy since it helps to broaden the efficacy against a range of genetically variable viral serotypes as well as making it more difficult for the virus to escape silencing through mutation. The value of this approach has been established in work with HBV (Romano et al., 2006), HIV (ter Brake et al., 2006), and coxsackievirus (Schubert et al., 2005). Here, we show that up to four different HBV-targeted miRNAs can be expressed and processed from a single pol II transcript and that the overall silencing potency of polycistronic plasmids is greater than that of monocistronic plasmids. Other groups have also reported silencing from plasmids that express multiple interfering RNAs, either as shRNAs or miRNAs (Chung et al., 2006; Sun et al., 2006; Xia et al., 2006). However, these groups used vectors that separated the individual miRNAs into different introns and/or co-expressed reporter proteins, design elements that are disadvantageous in clinical applications.

In addition to the ability to express multiple miRNAs from a single transcript, expression from a pol II promoter affords the opportunity for tissue-specific expression. We show that the hybrid mouse alpha-fetoprotein/albumin promoter from the pLIVE vector can direct silencing in liver derived Huh7 cells, but it is considerably less effective than the CMV promoter in HeLa cells. While we are pursuing alterations to this promoter for even better expression in human liver cells, we expect that by enhancing expression in the targeted tissue, we can improve the therapeutic profile of a silencing vector while reducing the need for tissue-specific uptake of systemically delivered plasmids.

A concern regarding the therapeutic use of interfering RNAs is their potential to induce an inflammatory response. It is becoming evident that the cellular response to these agents can vary, depending on cell type, sequence elements in the interfering RNA, mode of delivery, and chemical modifications to siRNAs (reviewed in (Sioud, 2006)). In general, however, interfering RNAs that are expressed and processed via endogenous cellular pathways appear to be least likely to induce a cellular response because they present molecular patterns recognized as "self" by cytoplasmic receptors and because they do not encounter cell surface or endosomal Toll-like receptors. Consistent with this, we found that the structured RNAs transcribed from our polycistronic plasmid do not significantly induce interferon response genes in HeLa cells. The very low level of induction that we did observe appears to arise from transfection with the plasmid itself and not from the plasmid-encoded miRNAs. Further work will be necessary to determine the response to these reagents in animal models of HBV infection.

Although the therapeutic potential of RNAi has been recognized for several years, challenges remain in its practical application. The vector design described here will help to maximize the efficacy of miRNAs by increasing their potency and range of targets by allowing multiple miRNAs to be produced from a single therapeutic vector. At the same time, the use of RNA pol II promoters can help in directing the therapeutic activity of these agents to specific cell types in an organism, even in the absence of precisely targeted delivery. Since the miRNA expression cassette is built into non-protein coding RNA, co-expression of a protein product is not necessary, and this is a desirable feature for therapeutic vectors. While we have designed our vector to target HBV RNAs, the general format of the miRNA expression cassette will be applicable in silencing additional cellular or disease-related genes.

Acknowledgements

The authors thank Daniel McCallus and Patrick Romano (Nucleonics, Inc.) for helpful discussions. This work was funded by a grant to LFS from Nucleonics, Inc., and by NIH grant AI053988.

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