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***Schistosoma mansoni* U6 gene promoter-driven short hairpin RNA induces RNA interference in human fibrosarcoma cells and schistosomes ★**

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Abstract

RNA interference (RNAi) mediated by short hairpin-RNA (shRNA) expressing plasmids can induce specific and long-term knockdown of specific mRNAs in eukaryotic cells. To develop a vector-based RNAi model for *Schistosoma mansoni*, the schistosome U6 gene promoter was employed to drive expression of shRNA targeting reporter firefly luciferase. An upstream region of a U6 gene predicted to contain the promoter was amplified from genomic DNA of *S. mansoni*. A shRNA construct driven by the predicted U6 promoter targeting luciferase was assembled and cloned into plasmid pXL-Bac II, the construct termed pXL-BacII_SmU6-shLuc. Luciferase expression in transgenic fibrosarcoma HT-1080 cells was significantly reduced 96 h following transduction with plasmid pXL-BacII_SmU6-shLuc, which encodes luciferase mRNA-specific shRNA. In a similar fashion, schistosomes of *S. mansoni* were transformed with the SmU6-shLuc or control constructs. Firefly luciferase mRNA was introduced into transformed schistosomes after which luciferase activity was analyzed. Significantly less activity was present in schistosomes transfected with pXL-BacII_SmU6-shLuc compared with controls. The findings revealed that the putative *S. mansoni* U6 gene promoter of 270 bp in length was active in human cells and schistosomes. Given that the U6 gene promoter drove expression of shRNA from an episome, the findings also indicate the potential of this putative RNA polymerase III dependent promoter as a component regulatory element in vector-based RNAi for functional genomics of schistosomes.

★Note: Nucleotide sequence data reported in this paper are available in the GenBank database under Accession Nos. [HQ677838](#), [HQ677839](#) and [HQ540317](#).

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Keywords

Schistosoma; Luciferase; RNA interference; U6 gene; Vector-based RNAi; Promoter

1. Introduction

Draft genomes of *Schistosoma japonicum* and *Schistosoma mansoni* were reported recently (*Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Berriman et al., 2009). New information in genome sequences will provide leads for development of new interventions for control and treatment of schistosomiasis (Brindley et al., 2009), which relies solely of the anthelmintic drug praziquantel at this time. Tools are in development to determine the importance of the new genomic sequences of schistosomes (Beckmann et al., 2007; Brindley and Pearce 2007; Kines et al., 2008; Mann et al., 2008), although there are difficulties with investigating the complex genomes of helminth parasites such as the schistosomes in contrast to model, free-living species. RNA interference (RNAi) is active in schistosomes, has been used to investigate increasing numbers of gene targets in most developmental stages, and is being optimized for high-throughput screens (Stefanic et al., 2010 and references therein).

Despite its utility, RNAi frequently leads to only transient gene silencing and, in addition, may be inaccessible to some developmental stages and/or tissues of schistosomes. In vivo, e.g. vector-based, RNAi approaches that lead to integration of transgenes encoding cassettes that express small interfering RNAs (siRNAs) can circumvent deficiencies with exogenous RNAi approaches by providing continuous and/or conditional gene silencing (ter Brake et al., 2006; Sliva and Schnierle, 2010). Recently, it has been demonstrated that pseudotyped murine leukemia virus (MLV) can be employed to transduce developmental stages of schistosomes, leading to chromosomal integration of retroviral transgenes and transgene reporter activity (Kines et al., 2008, 2010; Yang et al., 2010). Moreover, a vector-based RNAi approach has been reported in which the MLV transgene encoded a long hairpin RNA specific for a schistosome protease involved in hemoglobinolysis (Tchoubrieva et al., 2010).

In insect, mammalian, avian and some pathogenic protozoa, RNA polymerase III (Pol III) promoter-based DNA vectors can express siRNA or short hairpin RNA (shRNA) (Brummelkamp et al., 2002; Gou et al., 2003; Wakiyama et al., 2005; Linford et al., 2009). However, this has not been demonstrated in schistosomes. Here we describe a promoter-like element from the U6 gene of *S. mansoni*, a Pol III, non-coding RNA gene that is a component of eukaryotic spliceosome machinery (see Copeland et al., 2009). An episomal plasmid that included a U6 gene promoter driving a short hairpin transcript designed to target mRNA encoding firefly luciferase was constructed. After transfection of human fibrosarcoma cells and schistosomules with the construct, significantly less reporter luciferase activity was seen in the transformed human cells and schistosomes. These findings demonstrated the activity of a 270 bp promoter-like sequence of the schistosome U6 non-coding RNA gene to drive transcription of short transcripts from episomes delivered to both human cells and schistosomules of *S. mansoni*. The findings indicate the likely utility of this U6 gene promoter containing construct for vector-based RNAi approaches in schistosomes.

2. Materials and methods

2.1. Schistosomules

Biomphalaria glabrata snails infected with the NMRI (Puerto Rican) strain of *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD, USA.

Cercariae released from infected *B. glabrata* snails were mechanically transformed into schistosomules. Briefly, cercariae were concentrated by centrifugation (425 g/10 min) and washed once with schistosomule wash medium, RPMI 1640 supplemented with 1× penicillin, streptomycin, fungizone (1 × PSF) and 10 mM HEPES (Mann et al., 2010). Cercarial tails were sheared off by 20 passes through 22 gauge emulsifying needles after which schistosomule bodies were isolated from tails by Percoll gradient centrifugation (Lazdins et al., 1982). Schistosomula were washed three times in wash medium and cultured for up to 2 weeks at 37°C under 5% CO₂ in air in modified Basch's medium (Basch, 1981) supplemented with washed human erythrocytes, at a density of 1 µl packed red cells per ml of culture medium (Mann et al., 2010).

2.2. Isolation of a U6 gene promoter-like sequence

The sequence and predicted structure of the U6 small nRNA of *S. mansoni*, GenBank accession number L25920, have been described (Gu and Reddy, 1994; Copeland et al., 2009). The 109 nucleotides (nt) of the U6 RNA were employed as the query to search the Sanger Institute's *S. mansoni* shotgun reads (Sanger) database, (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni). An identical match was located to sequences in contig shisto3397e03.p1k. Flanking sequences upstream of the copy of the U6 gene in this contig were targeted for PCR, using primers SmU6F, 5'-TATCAGTGGTCTAGATGTATGCTTG-3', and SmU6R, 5'-GCTAATCTTCTCTGTATCGTTCCA-3'. The genome of *S. mansoni* includes between nine and 55 copies of the U6 gene (Copeland et al., 2009). Sequences (~474 bp) upstream and flanking the U6 gene as well as part of the 5'-terminus of the U6 gene were amplified from genomic DNA of *S. mansoni* (prepared as in Morales et al., 2007), cloned into the TOPO TA plasmid (Invitrogen) and the sequence identity confirmed to be identical to that in contig shisto3397e03.p1k.

2.3. Construction of shRNA expression vectors targeting firefly luciferase

The two-step PCR approach of Gou et al. (2003) and Linford et al. (2009) was utilized to construct plasmid vectors for in vivo episomal RNAi. The approach involves two rounds of PCR using one universal primer, specific for the promoter and two unique target sequences; the first oligonucleotide to add the sense strand of the shRNA, the second to add the loop region and anti-sense strand. For the hairpin sense strand sequence, we utilized 21 residues, nt 851–871, (5'-GTGCGTGCTGGTGCCAACCC-3'), of the firefly luciferase gene in pGL3 because this site and target sequence length have been demonstrated in earlier reports of episomal vector RNAi to lead to complete or near complete knockdown of luciferase activity in *Drosophila* Schneider 2 cells, NIH-3T3 mouse fibroblasts and L2 rat lung epithelia cells (Gou et al., 2003; Wakiyama et al., 2005). Two rounds of PCR were employed to generate the final shRNA constructs, using forward primer U₆SF₁, 5'-GCGCGCGGATCCGAGTGTATGTGCATTTGGTTG-3', and two reverse primers, Luc851R1, 5'-TCTCTTGAAGGGTTGGCACCAGCAGCGCACGGATTT CGCAC ATCACTAAC-3' and Luc851R2, 5'-GCGCGCCTCGAGAAAAAGTGCGTGCTGG TGCCAA CCCTCTCTTGAA-3'. The final PCR product comprised the *S. mansoni* U6 promoter, the sense strand of the shRNA hairpin, a loop TTCAAGAGA (9 bp) (Brummelkamp et al., 2002) region, the antisense strand of the hairpin, and the U6 termination sequence, TTTTT (Gou et al., 2003). In addition, *Bam*HI (GGATCC) and *Xho*I (CTCGAG) sites were introduced upstream of the promoter and after the TTTTT terminator, respectively, to facilitate cloning. As a control vector, we constructed a cassette where the 21 target residues (nt 851–871) of the luciferase gene were 'scrambled' as follows, 5'-ACCTACTGGGCAGGGAGCCGC-3', using the same forward primer (above) and the following reverse primers, ScramLuc851 R1, 5'-TCTCTTGAAG CCTACTGGGCAGGGAGCCGCAGGATTTTCGCACATCACTAAC-3'; ScramLuc851 R2,

5'-GCGCGCCTCGAGAAAAAGCGGCTCCCTGCCCAGTAG GTTCTCT TGAA-3'. Thermal cycling conditions and reaction volumes of Linford et al. (2009) were employed.

The final products were sized by agarose gel electrophoresis, eluted from the gel, cleaved with *Bam*HI and *Xho*I, and ligated into linearized plasmid pXL-BacII at the multiple cloning site within the transposon's inverted terminal repeats (Li et al., 2005). Top10 *Escherichia coli* cells (Invitrogen) were transformed with the ligation products and transformed colonies cultured on Luria-Bertani broth (LB) agar-ampicillin (100 µg/ml). Maxipreps of plasmid DNA were prepared from single bacterial colonies using PerfectPrep Endofree Maxi Kit 5Prime, Gaithersburg, MD, USA), and concentration and purity determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The nt sequences of the inserts of the vector RNAi constructs, termed pXL-BacII_SmU6-shLuc and pXL-BacII_SmU6-shScramLuc were confirmed, and assigned GenBank accession numbers [HQ677838](#) and [HQ677839](#), respectively.

2.4. Preparation of siRNAs, double stranded RNAs (dsRNAs) and firefly luciferase mRNA

Block-iT™ siRNA of 21 nt in length, specific for residues 851–871 of firefly luciferase (siLuc), was purchased from Invitrogen (Carlsbad, CA, USA). Long dsRNA specific for, and spanning the full-length luciferase coding sequence of 1,672 bp was synthesized by in vitro transcription from a template amplified by PCR from the firefly luciferase gene in pGL3 (Promega), using primers (F, 5'-TAA TAC GAC TCA CTA TAG GG T GCG CCC GCG AAC GAC ATT TA-3' and R, 5'-TAA TAC GAC TCA CTA TAG GGG CAA CCG CTT CCC CGA CTT CCT TA-3') tailed with the T7 promoter sequence). Synthesis and downstream purification of the dsRNA was accomplished using the Megascript RNAi kit (Ambion, Austin, TX, USA). Integrity of dsRNAs was verified visually after non-denaturing agarose gel (1%) electrophoresis. For preparation of firefly luciferase mRNAs (mLuc), in vitro transcriptions of capped RNAs from template PCR products were accomplished using the mMessage mMachine T7 Ultra kit (Ambion) (Correnti and Pearce, 2004; Rinaldi et al., 2008). dsRNA or mLuc was precipitated with 1 vol. of 5 M ammonium acetate and 2.5 vol. of 95% ethanol after which the precipitate was dissolved in water and concentration and purity determined, as above.

2.5. HT1080 cells expressing firefly luciferase

We employed a genetically modified HT1080 human fibrosarcoma cell line that constitutively expressed luciferase in these studies of vector-based RNAi. Primarily for other studies (S. Suttiaprapa, V.H. Mann and P.J. Brindley, unpublished data), we constructed lentiviral vectors using the ViraPower Gateway system (Invitrogen). In brief, pLenti6/R4R2/V5-DEST, which includes a gene conferring resistance to blasticidin, was modified by insertion of the promoter of the *S. mansoni* spliced leader RNA gene, upstream of the firefly luciferase gene (see Kines et al., 2006). The 293FT producer cells (Invitrogen) were transformed with the construct together with packaging plasmids delivered in liposomes (Invitrogen). Pseudotyped lentivirus expressing vesicular stomatitis virus (VSV) glycoprotein was harvested from culture media and concentrated by centrifugation (Sorvall SS-34 rotor) at 48,000 g for 90 min at 4°C. Pelleted virions were resuspended in Opti-MEM medium (Invitrogen) at 4°C, after which functional virion titers were determined using target HT1080 human fibrosarcoma cells (Invitrogen) in the presence of the antibiotic blasticidin (Invitrogen). To generate a stable HT1080 cell line expressing luciferase, HT1080 cells were transduced with lentivirus virions (titer, 10⁷ transducing units per ml) in the presence of polybrene (Sigma), and blasticidin-resistant cells, expressing luciferase, selected by culturing the cells in the presence of blasticidin for 14 days. Stable cells were isolated and cryopreserved and/or expanded for use as target, luciferase expressing HT1080 cells for the vector-based RNAi studies described here.

Luciferase expressing HT1080 cells were plated at a density of 5×10^5 cells/well in 6-well plates (Corning). Fifteen hours later, the cells were transfected with four or 10 μg of plasmid pXL-BacII_SmU6-shLuc or pXL-BacII_SmU6-shScramLuc or 10 pmol siLuc using the Lipofectamine 2000 transfection system (Invitrogen). A control group of cells exposed only to lipofectamine was included. One day later the cells were washed with PBS, after which plasmid transformed cells were incubated for 96 h in DMEM supplemented with 10% FBS, penicillin and streptomycin. Cells were counted and lysed in CCLR buffer (Promega) in order to determine levels of luciferase activity (see section 2.7).

2.6. Transfection of schistosomules with plasmids, siRNA and dsRNA

For the studies on RNAi in schistosomes, schistosomules were transfected with plasmid, dsRNA or siLuc by square wave electroporation. Specifically, schistosomules were transferred to cuvettes (4 mm gap, BTX, San Diego, CA, USA) containing media supplemented with 1 μg siLuc, 30 μg dsRNA or 20 μg of plasmid (pXL-BacII_SmU6-shLuc or pXL-BacII_SmU6-shScramLuc) and then subjected to a square wave pulse of 125 V of 20 ms duration, using a BTX ElectroSquarePorator™ ECM830, as previously described (Kines et al., 2010). Subsequently, the transfected worms were transferred to pre-warmed Basch's medium and maintained in culture for 48 h until mRNA encoding firefly luciferase was introduced into the cultured worms by electroporation. After electroporation, the schistosomules were maintained in culture for 3 h and then washed free of culture media, and wet pellets of worms snap frozen and stored at -80°C .

2.7. Luciferase activity assay

HT1080 cells and schistosomules were harvested, washed three times with schistosomule wash medium and stored as wet pellets at -80°C . Pellets of schistosomules were subjected to sonication (3 $\times$ 5 s bursts, output cycle 4, Misonix Sonicator 3000, Newtown, CT 06470, USA) in 300 μl of 1 $\times$ CCLR lysis buffer (Promega). Pellets of 1×10^6 HT1080 cells (cell numbers were determined by replicate counts of Trypan blue-stained cells using a hemacytometer) were lysed in 300 μl of 1 $\times$ CCLR lysis buffer. The lysates were clarified by centrifugation in a microfuge at 4°C , and analyzed for luciferase activity. Aliquots of 100 μl of clarified lysate were injected into 100 μl luciferin (Promega) at 25°C , mixed and the relative light units (RLUs) determined in a tube luminometer (Sirius, Berthold, Pforzheim, Germany) 10 s later (Correnti and Pearce 2004; Kines et al., 2010). Duplicate samples were measured, with results presented as the average of the readings of RLU/s/mg of soluble schistosome protein or, for HT1080 cells, as RLU/s/ 10^3 cells. Recombinant firefly luciferase (Promega) was included as a positive control. The protein concentration in the soluble fraction of the schistosome extract was determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL, USA).

2.8. Statistical analysis

Statistical comparisons were made by ANOVA. When significant differences were found among groups, a Student's *t*-test between controls and treatment conditions was applied, as appropriate. *P*-values of ≤ 0.05 were considered to be significant and *P*-values of ≤ 0.01 highly significant.

3. Results

3.1. Promoter region of the U6 non-coding RNA gene of *S. mansoni*

An identical match to the *S. mansoni* U6 gene was located in sequences in contig shisto3397e03.p1k of the draft genome assembly for *S. mansoni*. Flanking sequences upstream of the copy of the U6 gene in this contig were targeted for PCR (see section 2.2);

~474 bp upstream and flanking the U6 gene as well as part of the 5'-terminus of the U6 gene were amplified from genomic DNA of *S. mansoni*, cloned and sequenced (Fig. 1), therefore confirming the sequence identity as being identical to that in contig shisto3397e03.p1k. The 270 nt flanking and upstream of this copy of the *S. mansoni* U6 gene have been assigned GenBank accession number [HQ540317](#).

The putative *S. mansoni* U6 promoter and U6 gene were aligned with those from human (GenBank [X07425](#)) and mouse (GenBank [X06980](#)). The schistosome U6 non-coding RNA gene sequence is 96% identical to its human and murine orthologues. The sequence of the putative promoter upstream of the *S. mansoni* U6 gene diverged substantially from the mammalian sequences. Notwithstanding, we attempted to locate motifs characteristic of U6 gene promoters. The enhancer region, also known as the distal sequence element (DSE), consists of an octamer motif (OCT) and a *SphI* post-octamer homology (SPH) element. The core region comprises a proximal sequence element (PSE) and a TATA-like element (Dahlberg and Schenborn, 1988; Sturm et al., 1988; Schaub et al., 1999). Based on greater or lesser identity to the human and mouse motifs, we identified these prospective motifs of the enhancer and core regions in the 270 bp flanking the schistosome U6 gene; SPH element (21 residues), -258 ATTTGGTTGTTAAAAATATA -238; OCT (eight residues), -230 ATTACTAT -223; PSE (21 residues), -71 GTGCAAATGAGTGAATGAACG -51; and the TATA box (seven residues), -31 TATGATA -25. These motifs are annotated on the multiple sequence alignment of the U6 genes in Fig. 1.

3.2. U6 promoter is active in vector-based RNAi

We constructed plasmids pXL-BacII_SmU6-shLuc and pXL-BacII_SmU6-shScramLuc (Fig. 2A). The short hairpin predicted to be transcribed from the U6 promoter of this episome includes the 21 nt sense strand specific for residues 851–871 of the luciferase transcript, a loop of nine residues, the 21 residue antisense strand, and the UUUUU termination signal. The control construct pXL-BacII_SmU6-shScramLuc includes the same residues in the target strand, but the order of the residues was scrambled. First, we transfected HT1080 cells with the vector based RNAi constructs. A HT1080 cell line that stably expressed firefly luciferase was used for this experiment, with the aim of vector-based RNAi targeting the 'endogenous' luciferase. At 96 h after transfection with the vector RNAi plasmids or with siRNA, statistically significant knockdown of luciferase activity was evident. In particular, luciferase activity was reduced by 54.3% and 47.5% in HT1080 cells transfected with 4 µg or 10 µg of pXL-BacII_SmU6-shLuc, respectively, compared with untreated, control HT1080 cells (both $P \leq 0.01$), and by 30.2% in cells exposed to the siRNA transfected cells ($P \leq 0.05$). By contrast, no reduction in luciferase activity was evident in cells transfected with the control construct, pXL-BacII_SmU6-shScramLuc (Fig. 2B). Similar findings were observed in each of two biological replicates of this experiment. These results indicated that the putative *S. mansoni* U6 promoter drives short hairpin expression in a human cell line against an endogenously expressed reporter gene.

3.3. Schistosome U6 promoter-driven shRNA induces RNAi in schistosomules

The activity of pXL-BacII_SmU6-shLuc was examined in cultured schistosomules. At 48 h after transfection with the vector RNAi plasmid, or with dsRNA or siRNA, statistically significant knockdown of luciferase activity was evident. Specifically, luciferase activity was reduced by 47.5% in schistosomules transfected with 20 µg of pXL-BacII_SmU6-shLuc compared with schistosomules treated with 20 µg of the control plasmid, pXL-BacII_SmU6-shScramLuc (183,030.5 versus 348,651.3 RLU/s/mg) ($P \leq 0.05$) (Fig. 3). The RNAi induced with dsRNA or siRNA was even greater than that with pXL-BacII_SmU6-shLuc, both > 95% knockdown of luciferase activity compared with the worms transfected with the control plasmid, pXL-BacII_SmU6-shScramLuc ($P \leq 0.01$). More luciferase activity was seen in

schistosomules not transfected with any nucleic acids ($P \leq 0.01$), a control group that was termed 'mock' (Fig. 3). Similar findings were observed in three of four replicates of the experiment.

4. Discussion

RNAi is a widely employed approach in reverse genetics for specific knockdown of target genes. The RNAi mechanism is conserved among eukaryotes, including schistosomes (see Krautz-Peterson et al., 2010). RNAi is triggered by dsRNA and results in inactivation of the target gene through degradation of homologous mRNAs and/or inhibition of mRNA translation. In the context of vector-based RNAi, the molecular biology of RNAi has been reviewed (e.g., Cheng and Chang, 2007; Sliva and Schnierle, 2010). In brief, episomal or integrated transgenes, comprising pol III promoters driving target sequence-specific cassettes, lead to over-expression of shRNAs, of ~21 nt of stem (range 19–29 nt) and ~9 nt (range 4–23) of loop sequences (Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002). After export from the nucleus the shRNA is cleaved by Dicer and enters the RNA-induced silencing complex, which unwinds the RNA and targets the specific mRNA for degradation. Vector-based RNAi provides advantages over the direct introduction of siRNA or dsRNA to target cells, including long-term and/or conditional gene silencing. It also can be cost effective, given that synthesis of siRNA is expensive, its effect transient and in addition, long dsRNA can induce a cytotoxic response in mammalian cells (Williams, 1999). On the other hand, the RNAi effect using a vector-based system is more stable due to sustained production of shRNA.

We isolated 270 bp of genomic DNA flanking the *S. mansoni* U6 small nRNA gene, a component of the spliceosome machinery (see Copeland et al., 2009), and constructed a RNAi expression cassette using this putative promoter to drive expression of shRNAs specific for firefly luciferase. We observed significant knockdown of luciferase in human fibrosarcoma cells and in schistosomules transfected with the expression cassette. The findings demonstrated that the *S. mansoni* U6 gene promoter drove shRNA expression that induced RNAi in a human cell and in schistosomules. In addition to schistosome and human U6 gene promoters, orthologous U6 promoters from pathogenic amebae, *Drosophila*, sea squirts, fugu fish, chicken and bovines also function in Pol III driven vector-based RNAi expression systems (Lambeth et al., 2005; Wakiyama et al., 2005; Wise et al., 2007; Nishiyama and Fujiwara, 2008; Zenke and Kim, 2008; Linford et al., 2009).

Although this *S. mansoni* U6 gene promoter is active in schistosomes and in HT1080 cells, the knockdown experiments performed in HT1080 cells were more consistent and reproducible than those with schistosomules. However, even in HT1080 cells we did not observe a concentration effect; 10 μ g did not induce significantly greater silencing than 4 μ g of pXL-BacII_SmU6 shLuc. This may reflect saturation of the cellular microRNA/shRNA pathway leading to competition for limited cellular factors such as nuclear karyopherin exportin-5 (see Grimm et al., 2006; Snøve and Rossi, 2006). The non-specific reduction of luciferase activity in schistosomules detected in the control shRNA group may reflect dysfunction of the mRNA translation machinery. Schistosomules clearly are less tractable for these approaches than mammalian cell lines but since functional genomics tools are needed for schistosomes, and because cell lines are not available for schistosomes, we have targeted larval schistosomes here to investigate a prospective new approach (Mann et al., 2008, 2010). Cultured HT1080 cells not only have a larger surface area, but here were transfected only once with the vector short hairpin plasmid whereas the schistosomules were transfected with this plasmid and, secondly, with the mRNA encoding luciferase. Exposing worms twice to electroporation may be deleterious and reflected in inconsistent outcomes in the replicates. Moreover, knockdown of luciferase in these particular HT1080 cells

represented silencing of an 'endogenous' gene since the cells expressed firefly luciferase from integrated lentiviral transgenes. Perhaps most critical was that since schistosomules are multicellular, some but not all of the cells were transformed with both pXL-BacII_SmU6-shLuc and luciferase mRNA. Cells of the digestive tract and/or tegument are likely transduced by the plasmid and/or the mRNA, whereas more internal cells may not have been transfected. Nonetheless, we can predict that after entry into the schistosome cell, the episome was transcribed and processed, and the siRNAs transmitted to adjacent cells by transporters such as Sid 1 (Krautz-Peterson and Skelly, 2008). In this regard and to improve assay reproducibility, increasing the time between the plasmid and the luciferase mRNA transfection might be beneficial so that siRNAs might spread from the transfected cells, leading to enhanced knockdown.

Recently, proof-of-principle for vector-based RNAi was demonstrated in *S. mansoni*, using a MLV (retrovirus) vector (Tchoubrieva et al., 2010). However, those investigators used a RNA polymerase II dependent promoter and a long hairpin of ~120 bp targeting a protease involved in hemoglobinolysis. Whereas this retroviral vector-based RNAi system is functional, there may be advantages to deploying a Pol III dependent promoter: the U6 promoter is known to competently and continuously transcribe small RNAs, is highly active in all or most cells and tissues, and its diminutive size fits within the limits on cargo size of the expression cassette (Paddison et al., 2002; Scherr and Eder, 2007). The significance of both findings with vector-based RNAi in schistosomes, the present findings and those of Tchoubrieva et al. (2010) is that vector-based RNAi should allow targeting of any schistosome gene for continuous knockdown and subsequent examination of the importance of the targeted gene. Moreover, plasmid pXL-BacII, from which we constructed pXL-BacII_SmU6-shLuc, contains the inverted terminal repeats of transposon piggyBac (Li et al., 2005). Given that piggyBac is transpositionally active in *S. mansoni* (Morales et al., 2007), pXL-BacII_SmU6-shLuc could be utilized to generate transgenic schistosomes, that inherit trans-generational silencing of report luciferase, by co-transfecting schistosomes with this shRNA inducing plasmid along with the piggyback transposase.

High throughput RNAi analysis will advance functional genomics for schistosomes, and indeed parasitic helminths at large. We anticipate that the information presented here describing the schistosome U6 promoter and its activity in vector-based RNAi will contribute to establishment of these analyses. We are interested to establish long-term, stable and trans-generational RNAi to aid discovery of essential schistosome genes which could, in turn, be targeted in new approaches for treatment and control of schistosomiasis.

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Fig. 1.

Multiple sequence alignment of the U6 genes of *Schistosoma mansoni*, *Homo sapiens* and *Mus musculus*. The multiple alignment was assembled with ClustalW (Thompson et al., 1994) using BioEdit, version 7.0.5 (Hall, 1999) and the box shade feature of GeneDoc (Nicholas and Nicholas, 1997, Pittsburgh Supercomputing Center, Pittsburgh, PA 15213, USA), and the following sequences: *S. mansoni* (GenBank [L25920](#) (coding region) and [HQ540317](#) (upstream flanking region)); human, (GenBank [X07425](#)), mouse (GenBank [X06980](#)). Residue one (black, curved arrow) is the first nucleotide (nt) of the U6 RNA gene sequence. Characteristic motifs of the enhancer and core regions of the U6 gene promoter are identified or predicted (red colored rectangles). The enhancer distal sequence element includes an octamer motif (OCT) and *SphI* post-octamer homology (SPH) element and the core region comprises a proximal sequence element (PSE) and a TATA-like element. Consensus sequences are indicated under the PSE and OCT motifs. Note that the relative positions for the SPH and OCT elements are reversed in mouse U6.

Fig. 2.

Episomal vector-based RNA interference (RNAi) of firefly luciferase activity in human fibrosarcoma HT1080 cells. A) Schematic illustration of the insert of plasmid of pXL-BacII_SmU6-shLuc, including the inverted terminal repeats (ITR) flanking the short hairpin (sh)RNA cassette, the *Schistosoma mansoni* U6 gene promoter, the sense strand (21 nucleotide (nt), residues 851–871 of the gene encoding firefly luciferase, the nine residue loop, the 21 nt anti-sense strand and the TTTTTT terminator residues. The predicted shRNA is shown below the plasmid construct. B) Luciferase activity in HT1080 cells expressed as relative light units (RLU)/s/10³ cells. HT1080 cells were transfected with 1 μg of small interfering RNA of 21 nt in length, specific for residues 851–871 of firefly luciferase (siLuc) ; 4 μg pXL-BacII_SmU6-shLuc (shLuc_4μg); 10 μg of pXL-BacII_SmU6-shLuc (shLuc_10μg); 4 μg plasmid pXL-BacII_SmU6-shScramLuc (shScrambled_4μg); 10 μg of control plasmid pXL-BacII_SmU6-shScramLuc (shScrambled_10μg); and no treatment control (mock). Bars are ± S.D. (*n* = 2). Significant differences between treated groups and the (mock) control are indicated: *, *P* ≤ 0.05; **, *P* ≤ 0.01.

Fig. 3.

Episomal vector-based RNA interference (RNAi) of firefly luciferase activity in 1 day old schistosomules. Luciferase activity in schistosomules expressed as relative light units (RLU)/s/mg of protein. One day old schistosomules were electroporated in the absence of plasmid or RNAs (mock), 30 μ g of firefly luciferase double stranded RNA (dsLuc), 10 μ g of small interfering RNA of 21 nt in length, specific for residues 851–871 of firefly luciferase (siLuc), 20 μ g of control plasmid pXL-BacII_SmU6-shScramLuc (ShScram) and 20 μ g of plasmid pXL-BacII_SmU6-shLuc (shLuc). Bars are $\pm$ S.D. ($n = 2$). Significant differences between treated groups and the (mock) control are indicated: *, $P \leq 0.05$; **, $P \leq 0.01$.