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Human U6 promoter drives stronger shRNA activity than its schistosome orthologue in *Schistosoma mansoni* and human fibrosarcoma cells

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Abstract

Blood flukes or schistosomes are the causative agents of human schistosomiasis, one of the major neglected tropical diseases. Draft genome sequences have been reported for schistosomes, but functional genomics tools are needed to investigate the role and essentiality of the newly reported genes. Vector based RNA interference can contribute to functional genomics analysis for schistosomes. Using mRNA encoding reporter firefly luciferase as a model target, we compared the performance of a schistosome and a human promoter from the U6 gene in driving shRNA in human fibrosarcoma cells and in cultured schistosomes. Further, both a retroviral (Murine leukemia virus [MLV]) and plasmid (*piggyBac*, pXL-Bac II) vector were utilized. The schistosome U6 gene promoter was 270 bp in length, the human U6 gene promoter was 264 bp; they shared 41% identity. Following transduction of both HT1080 fibrosarcoma cells and schistosomules of *Schistosoma mansoni* with pseudotyped MLV virions, stronger knockdown of luciferase activity was seen with the virions encoding the human U6 promoter driven shRNA than the schistosome U6 promoter. A similar trend was seen after transfection of HT1080 cells and schistosomules with the pXL-Bac-II constructs – stronger knockdown of luciferase activity was seen with constructs encoding the human compared to schistosome U6 promoter. The findings indicate that a human U6 gene promoter drives stronger shRNA activity than its schistosome orthologue, not only in a human cancer cell line but also in larval schistosomes. This RNA polymerase III promoter represents a potentially valuable component for vector based RNA interference studies in schistosomes and related platyhelminth parasites.

Keywords

Schistosome; *Schistosoma mansoni*; short hairpin RNA; U6 gene; promoter; firefly luciferase; RNA interference; murine leukemia virus; *piggyBac* transposon

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Introduction

Vector-based RNAi approaches that lead to chromosomal integration of transgenes encoding cassettes expressing small interfering RNAs (siRNAs) can circumvent deficiencies with cytoplasmic RNAi approaches by providing long-term, continuous and/or conditional gene silencing (ter Brake et al. 2006; Moore et al. 2010; Sliva and Schnierle 2010). By contrast, delivery of dsRNAs/ siRNAs from the exterior of the cell to the cytoplasm frequently delivers only transitory gene silencing, may be inaccessible to some developmental stages and/or tissues, can be expensive and, in addition, cytoplasmic dsRNAs may induce apoptosis and interferon signaling responses in some situations (Haasnoot et al. 2007; Sliva and Schnierle 2010; Handa et al. 2011).

The schistosomes are considered the most important of the human helminth parasites in terms of the morbidity and mortality which they cause (Hotez et al. 2008; Han et al. 2009; Rollinson 2009; Gurarie et al. 2011). In addition, *Schistosoma haematobium* is a cause of squamous cell carcinoma of the bladder and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (Bouvard et al. 2009). Following the recent reports of the draft genome sequences of two of the major human schistosome species (Berriman et al. 2009; *Schistosoma japonicum* Genome Sequence and Functional Analysis Consortium, 2009), efforts have increased towards harnessing the functional genomics approaches widely used in model species and human gene therapy for investigation of the importance and essentiality of these new schistosome genes (Stefanic et al. 2010; Collins et al. 2011). Information obtained with these advances can be expected to guide development of new interventions for these major neglected tropical disease pathogens (Brindley et al. 2009; Han et al. 2009).

In relation to this interest in expanding functional genomics for schistosomes, it has been demonstrated that both pseudotyped murine leukemia virus (MLV) and the transposon *piggyBac* can transduce developmental stages of schistosomes, leading to chromosomal integration of retroviral transgenes and transgene reporter activity (Morales et al. 2007; Kines et al. 2008; Yang et al. 2010; Rinaldi et al. 2011). Moreover, vector-based RNAi approaches have been reported in schistosomes (e.g. Zhao et al., 2008). The goal of the present investigation was to compare and contrast the performance of two U6 gene promoters, one from *S. mansoni*, the other from the human genome, in driving short hairpin RNAs in cultured schistosomes. There may be advantages to deploying a RNA polymerase (Pol III) promoter: the U6 promoter is known to competently and continuously transcribe small RNAs, is highly active in all or most cells and tissues where this has been examined (information on this aspect is not available for schistosomes, so it is not known whether Pol III is likewise widely active in cells of this flatworm), and its diminutive size fits within the limits on cargo size of the expression cassette (Paddison et al. 2002; Scherr and Eder 2007). Defining active promoter elements for vector based RNAi in schistosomes can be expected to be worthwhile since studies of the human schistosomes are more problematic than established approaches with cultured mammalian cells or with more well studied model species such as *Caenorhabditis elegans* and *Drosophila melanogaster*. The present findings revealed the superior performance of a human U6 promoter when compared to an endogenous U6 promoter from *S. mansoni* in driving active shRNAs in cultured schistosomes.

Material and Methods

HT1080 fibrosarcoma cells expressing firefly luciferase

We employed a genetically modified HT1080 human fibrosarcoma cell line, that constitutively expresses luciferase, in these studies of short hairpin RNA (Ayuk et al. 2011). In brief to generate a HT1080 cell line stably expressing luciferase, HT1080 cells (ATCC, Manassas, VA) were transduced with lentivirus virions (ViraPower Promoterless Lentiviral Gateway Expression System, Invitrogen) that includes a gene conferring resistance to blasticidin, and the firefly luciferase gene driven by the *S. mansoni* spliced leader RNA gene promoter (see Kines et al. 2006) in the presence of polybrene (Sigma). Blasticidin-resistant cells expressing luciferase were selected by culturing in the presence of blasticidin for 14 days. Stable cells were isolated, cryopreserved and/or expanded for use as target, luciferase expressing HT1080 cells for the vector based RNAi (below).

Schistosoma mansoni—*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. In brief, cercariae were concentrated by centrifugation (425g/10 min) and washed once with schistosomule wash medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 units/ml of penicillin, 200 µg/ml of streptomycin, 500 ng/ml of amphotericin B and 10 mM HEPES). Cercarial tails were sheared from the larvae 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 schistosomule wash medium and cultured in Basch's medium (Basch 1981) at 37 °C under 5% CO₂ in air for up to 14 days. Some cultures were supplemented with washed human erythrocytes, at a density of one µl of packed red cells per ml of medium (Mann et al. 2010).

Construction of shRNA expression cassettes targeting firefly luciferase

The sequence of the human U6 small nuclear RNA gene (GenBank X07425) was used to design primers HsU6F and HsU6R (Table S1) and the primers employed to amplify upstream and part of the 5'-terminus of the U6 gene were amplified from genomic DNA from human white blood cells (denoted by a co-author of this report) using a kit (ENZA, Omega Bio-tek, Doraville, GA). The amplicon was ligated into pCR4 TOPO (Invitrogen), the insert sequenced, the nucleotide sequence aligned with other human and schistosome U6 promoters, and this human U6 promoter sequence included in the shRNA expression cassette in the MLV pLNHXΔD70 (generated by the removal of *Drosophila* heat shock 70 gene promoter from pLNHX using *Xho* I) (Suttiapra, unpublished) and *piggyBac* (pXL-BacII) constructs assembled here (Figure 1). The *S. mansoni* U6 promoter has been described (Ayuk et al. 2011).

A two-step PCR approach was performed to construct the shRNA expression cassettes targeting firefly luciferase (Gou et al. 2003; Linford et al. 2009; Ayuk et al. 2011) that included either a human or a schistosome U6 promoter. The approach involved two rounds of PCR using one universal primer, specific for the promoter (human or schistosome) and two unique target sequences, the first oligonucleotide to add the sense strand of the shRNA, the second to add the loop region, anti-sense strand and terminal thymidines. For the hairpin sense strand, we included 21 residues, nucleotides 851-871, 5'-GTGCGCTGCTGGTGCCAACCC-3', of the firefly luciferase (cDNA) gene in pGL3 (Promega) because this site and target sequence length have been demonstrated in shRNA studies to lead to complete or near complete knockdown of luciferase activity in mammalian and insect cell lines (Gou et al. 2003; Wakiyama et al. 2005). For a control shRNA, we

constructed cassettes where the 21 target residues 851-871 of the luciferase gene were scrambled, as follows, 5'-ACCTACTGGGCAGGGAGCCGC-3', using the same forward primer (above) and the reverse primers (Table S1). The final products were sized by agarose gel electrophoresis, eluted from gel, cleaved with *Xho*I for pLNHX and *Bam*H I and *Xho*I for pXL-Bac II (Li et al. 2005), and ligated into linearized retroviral or transposon vector plasmids. Figure 1 provides an illustration of constructs, termed pLNHX_SmU6_shLuc (GenBank accession JN255692), pLNHX_HsU6_shLuc (JN255690), pLNHX_HsU6_ScramLuc (JN255691), pXL-BacII_SmU6-shLuc (HQ677838), pXL-BacII_SmU6-shScramLuc (HQ677839), pXL-BacII_HsU6-shLuc (JF298906), and pXL-BacII_HsU6-shScramLuc (JF298907). Sequences of oligonucleotide primers used in assembly of these shRNA expression cassettes are provided in Table S1.

Production of pseudotyped retroviral virions

Plasmid constructs, prepared with a maxiprep kit (5 Prime, Gaithersburg, MD) were employed to produce the virions as described (Kines et al. 2010; Rinaldi et al. 2011). In brief, GP2-293 cells were co-transfected with the MLV encoding plasmid (Figure 1) along with pVSVG, which encodes vesicular stomatitis virus glycoprotein (the pseudotyping envelope) using liposomes (Lipofectamine 2000, Invitrogen). Culture supernatants were collected 48 hours later, filtered through 0.45 μ m pore size membranes, the filtrate incubated with DNase I (New England Biolabs, Ipswich, MA) to remove contaminating plasmids, and virions in culture supernatants recovered by centrifugation (Sorvall SS34 rotor, 50,000 g, 90 minutes, 4°C). Pelleted virions were resuspended in schistosomule wash medium (above), virion titers estimated using quantitative PCR (Mann et al. 2011), and aliquots of virions stored at -80°C until needed. pLNHX Δ D70 was employed to produce control virions without shRNA encoding cassettes.

Messenger RNA encoding firefly luciferase

For preparation of firefly luciferase mRNAs (mLuc), *in vitro* transcriptions of capped RNAs from template PCR products from the luciferase gene in pGL3-Basic (Promega) were accomplished using the mMessage mMachine T7 Ultra kit (Ambion) (Correnti and Pearce 2004). mLuc was precipitated with one volume of 5 M ammonium acetate and 2.5 volumes of 95% ethanol after which the precipitate was dissolved in water and concentration and purity determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Transduction of HT1080 cells

Luciferase expressing HT1080 cells were plated at a density of 2 to 5 $\times 10^5$ in 6-well (35 mm) plates to achieve 30 to 50% confluence the following day. For transduction with retroviral virions, pLNHX_HsU6_shLuc or pLNHX_HsU6_Scram control virions (6.5×10^8 virions) were added to the cells which were then cultured for 18 h in 2 ml of DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 μ g/ml of streptomycin and 8 μ g/ml polybrene. Culture media were changed the following day. The transduced cells were harvested at 96 hours after addition of virions. For some experiments, transduced HT1080 cells were subjected to selection on G418 at 500 μ g/ml for 10 days, since the retroviral encodes the neomycin resistance gene (Figure 1), with changes of culture media every second day. After harvesting, cells were counted and lysed in CCLR buffer (Promega) in order to determine levels of luciferase activity (below).

In like fashion, HT1080 cells at ~90% confluence were transfected with 4 μ g of plasmid pXL-BacII_SmU6-shLuc, pXL-BacII_SmU6-shScramLuc, pXL-BacII_HsU6-shLuc or pXL-BacII_HsU6-shScramLuc using Lipofectamine 2000. A control group of cells not transfected with plasmids but exposed to the liposomes was included (mock). The following

day, cells were washed with 1X PBS, and then cultured for 96 h in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin. Cells were counted and lysed, as above.

Transduction of schistosomules

For transduction of schistosomes, virions – at titers ranging from 2.6×10^9 to 1.5×10^{10} per treatment well, with specific titers noted in figure legends, below - were added to cultures of seven day old schistosomules, 10^3 - 10^4 schistosomules per well (24-well plates; Corning) in one ml schistosomule media containing 8 µg/ml polybrene. Media were changed at 18 h after addition of virions, after which schistosomules were maintained in culture for three days. At this point these schistosomules were transfected with mRNA encoding firefly luciferase. Three hours after electroporation the parasites were harvested for luciferase activity assays. In some experiments, the schistosomules were exposed to the virus by centrifugation (800 g, 60 min, 21°C) in the presence of polybrene.

Schistosomules were transfected with pXL-Bac II constructs by square wave electroporation; the worms were transferred to cuvettes (4 mm gap, BTX, San Diego, CA, USA) containing media supplemented with 20 µg of plasmid (pXL-BacII_SmU6-shLuc or pXL-BacII_SmU6-shScramLuc or pXL-BacII_HsU6-shLuc or pXL-BacII_HsU6-shScramLuc) and then subjected to a square wave pulse of 125 V of 20 ms duration, using a BTX ElectroSquarePorator™ ECM830, as described (Kines et al. 2010). Transfected worms were transferred to pre-warmed Basch's medium and maintained in culture for 48 hours. mRNA encoding firefly luciferase was introduced into the cultured worms by electroporation (using the same conditions as for the plasmids). 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. (It is pertinent to note that exposing worms twice to electroporation may be deleterious, and reflected in inconsistent outcomes in the replicates [Ayuk et al., 2011].)

Luciferase activity assay

HT1080 cells and schistosomules were harvested, lysed, clarified supernatant (after centrifugation of lysates, 10,000g, 10 min 4° C), analyzed for luciferase activity. Aliquots of 100 µl of 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 seconds later (Correnti and Pearce 2004). Replicate samples were measured, with results presented as the average of the readings of relative light units (RLU) per second per µg of soluble HT1080 cells or schistosome protein. Recombinant firefly luciferase (Promega) was included as a positive control in order to verify that the luciferin substrate and luminometer were functioning in a normal, expected fashion. Protein concentration of soluble fractions of transduced HT1080 cell and schistosome extracts were determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL).

Results

U6 promoters diverge between schistosomes and humans

Whereas it was clear from a recent study that a U6 promoter from *S. mansoni* drove shRNA expression (Ayuk et al. 2011), here we compared the performance of a human U6 promoter with a schistosome orthologue since the human U6 promoter is used widely in shRNA studies (Makinen et al. 2006; Roelz et al. 2010), including its inclusion as the promoter in the commercial vector pENTR/U6 from Invitrogen. Several U6 genes from the human genome are active although it appears that the promoter termed U6-1 is the widely utilized variant (Kunkel and Pederson 1988; Domitrovich and Kunkel 2003) (GenBank X07425).

We aligned the X07425 sequence, the human U6 promoter from pENTR/U6 (here termed Invitrogen_U6), and GenBank JN255693, a variant of the human U6 promoter we cloned and sequenced from the blood donated by a co-author of this report, and a *S. mansoni* U6 gene promoter (HQ540317) (alignment not shown but available from corresponding author). The schistosome U6 gene promoter was 270 bp in length, the human U6 gene promoter was 264 bp, and they shared 41% identity. By contrast, the three human orthologues aligned were nearly identical; X07425 differed from JN255693 and Invitrogen_U6 by the inclusion of an additional residue, a thymidine (T) at position-98. This T occurred outside conserved Pol III binding domains, SPH, OCT, PSE and TATA, which were identical in the three human sequences. However, clear differences were apparent in all four domains between the schistosome (HQ540317) and human U6 promoters (Ayuk et al., 2011). The human U6 gene promoter in GenBank JN255693 was included as the promoter in shRNA expression cassettes constructed here (Figure 1).

Human U6 promoter drives shRNA activity in schistosomes

At the outset, we examined the human U6 promoter in the context of transduction of schistosomules by pLNHX based virions. Human fibrosarcoma HT1080 cells, stably expressing luciferase, and seven day old schistosomules were transduced with retroviruses encoding the shRNA targeting firefly luciferase or a scrambled sequence (control). As shown in Figure 2, in both the HT1080 cells (panel A) and the schistosomes (panel B), which had been transfected with firefly luciferase encoding mRNA (mLuc), substantial knockdown of luciferase was apparent in the target HT1080 (47% knockdown) and schistosome parasites (81%) (Figure 2, panel A (HT1080 cells), and panel B (schistosomules) when the effect of the pLNHX_HsU6-shLuc and pLNHX_HsU6_Scram virions was compared. Similar findings were seen when transduced HT1080 cells were selected on geneticin/G418 for 10 days (49%) (Figure S1, panel A). Also, similar results were seen with schistosomules at 10 days after transduction by virions (60%) and in schistosomules exposed to the virus by centrifugation (85%) (Figure S1, panels B and C, respectively).

Following retroviral transduction, human U6 drives stronger shRNA than schistosome U6 in schistosomules

Having determined above that the heterologous human U6 promoter was active in shRNA cassettes in schistosomes, we next compared the performance of the schistosome and human U6 promoters in schistosomes. Groups of schistosomules were transduced with pLNHX_HsU6-shLuc, pLNHX_SmU6-shLuc virions, or control pLNHX Δ D70 virions. An additional control group, the mock transduction group exposed only to polybrene, was included. In the experiment presented in Figure 3, a knockdown of 38% luciferase activity was observed in schistosomules transduced with pLNHX_HsU6-shLuc virions compared to both mock and pLNHX Δ D70 control groups. By contrast, no knockdown was observed in worms transduced with the pLNHX_SmU6-shLuc virions. This same trend was observed in two replicates of this experiment (not shown). Thus, the human U6 promoter drove stronger shRNA activity than its schistosome orthologue in *S. mansoni*. Indeed, the schistosome promoter was barely or not active under these conditions.

Human U6 promoter drove stronger shRNA activity than schistosome U6 promoter after delivery of transgenes in a transposon plasmid

In addition to MLV retroviral based transgenesis of schistosomes and HT1080 cells to compare the human and schistosome U6 promoters, we carried out parallel studies using the *piggyBac* transposon plasmid pXL-Bac II (Figure 1). As above, we targeted HT1080 cells expressing luciferase and schistosomules transfected exogenously with luciferase encoding mRNA (mLuc). First, in each target type, the promoter driving the shRNA targeting

luciferase produced a strong knockdown of luciferase in contrast to the control constructs targeting the scrambled sequence (Figure 4, panels A and B). In both HT1080 and in schistosomules, the human U6 promoter delivered greater knockdown of luciferase activity than the schistosome U6 promoter; 88 % vs. 75% in HT1080, 80% vs. 35% in schistosomules. By contrast, similar levels of luciferase activity were evident in cells (or schistosomes) transfected with the control constructs, pXL-BacII_SmU6-shScramLuc (SScram) or pXL-BacII_HsU6-shScramLuc (HScram). Similar findings were seen on each of two occasions these assays were performed (Figure S2). (Luciferase levels in the mock i.e. no shRNA control were somewhat higher than in cells (or schistosomules) transfected with the shRNA control plasmids, a phenomenon we have seen and discussed previously [Ayuk et al., 2011].)

Discussion

Deployment of shRNA for RNA interference represents a new strategy for functional genomics analyses of schistosomes and the human helminth parasites at large, which are major neglected disease pathogens. Whereas this is an emerging area, three recent reports have shown that both Pol II and Pol III promoters drive short hairpins in *Schistosoma japonicum* and *S. mansoni* that can silence target genes (Zhao et al. 2008; Tchoubrieva et al. 2010; Ayuk et al. 2011). The findings revealed that endogenous genes - *mag0 nashi* in *S. japonicum* and cathepsin B in *S. mansoni*, as well as reporter firefly luciferase, can be silenced following transfection of the schistosomes with transgene cassettes encoding cognate hairpin RNAs. Furthermore, endogenous - *S. mansoni* actin (Tchoubrieva et al., 2010) and *S. mansoni* U6 (Ayuk et al., 2011) - and a heterologous promoter - human H1 (Zhao et al., 2008) - were shown to be active.

Here endo- and exogenous orthologues of U6 gene promoters were examined for performance in shRNA expression cassettes in *S. mansoni*. Promoters from the genomes of both *S. mansoni* and *Homo sapiens* were tested as components of the shRNA cassettes. The genomes of both humans and schistosomes include numerous copies of the U6 small nuclear RNA gene: there are at least nine copies of the human U6 gene, five of which are active (Domitrovich and Kunkel 2003), and nine to 55 copies of the *S. mansoni* orthologue (Copeland et al. 2009). The copy of the human U6 gene promoter tested here appeared to be identical or close to identical to U6-1 (Domitrovich and Kunkel 2003), a well-characterized copy that efficiently drives shRNAs in human cells and cells from non-congruent species (Boden et al. 2003; Roelz et al. 2010). We employed both pseudotyped retrovirus (MLV) and *piggyBac* based vectors, both known to integrate transgenes into chromosomes of this platyhelminth parasite (Morales et al. 2007; Kines et al. 2008). Notably, the findings revealed that the human U6 promoter displayed superior performance to the schistosome U6 orthologue: in both the retroviral and donor transposon contexts, the human U6 promoter drove stronger knockdown of firefly luciferase in a human fibrosarcoma cell line and in cultured schistosomes.

The promoters were of similar length, 264 nt for human U6 and 270 nt for *S. mansoni*, but were only 41% identical. The binding motifs for Pol III in the human U6 promoter have been characterized (Domitrovich and Kunkel 2003). They include an enhancer region, also termed the distal sequence element (DSE). The DSE consists of an octamer motif (OCT) and a *Sph* I post-octamer homology (SPH) element. In addition, a 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; Myslinski et al. 2001). Based on greater or lesser identity to the human and mouse motifs, previously we identified these prospective motifs of the enhancer and core regions in the 270 bp upstream of a schistosome U6 gene (Ayuk et al. 2011). It is feasible that clear differences in sequence in the PSE and other domains - for

example, TATGATA versus TATATA for the TATA motif; ATTACTAT versus ATTTGCAT for the OCT motif in the schistosome and human promoters, respectively (Ayuk et al. 2011) - were reflected in the differential activity of the human and schistosome U6 gene promoters. However, confirmation of the influence of these motif differences of the schistosome and human U6 promoters in schistosomes awaits further investigation. In addition, other copies of the *S. mansoni* U6 promoter may exhibit sequence differences that drive stronger shRNA expression, as has been demonstrated among the human U6 gene orthologues (Domitrovich and Kunkel 2003).

The present results extend earlier findings wherein it was demonstrated the schistosome U6 promoter drove shRNA in both targets - HT1080 fibrosarcoma cells and blood stage schistosomes. However, based on these findings, we recommend deployment of the human U6 promoter rather than the schistosome promoter in future shRNA investigations. Zhao et al (2008) demonstrated the activity of the human H1 gene promoter for shRNAs in *S. japonicum*. Makinen et al (2006) demonstrated that the human U6 was more efficient than the H1 promoter in silencing reporters in both human (293T) and murine (c166-GFP) cell lines, leading to 80% knockdown of the transcript encoding green fluorescent protein. Similar trends were apparent *in vivo*, in mouse brains. (H1 RNA is the RNA component of the human nuclear RNase P; the H1 promoter, which like that of U6 is a type 3 RNA polymerase III promoter, is unusually compact [Myslinski et al., 2001].) Moreover, shRNA expression from the human U6 promoter resulted in a four-fold increase in knockdown efficiency compared to expression from the mouse U6 promoter in human and in mouse cell lines (Roelz et al. 2010). Accordingly, the human U6 promoter appears in general to drive strong shRNA based knockdown; the present findings demonstrated that this also occurs in schistosomes. In less well studied helminth parasites, it is reasonable to expect the human U6 gene would also be serviceable for shRNA investigations. Indeed, deploying the human U6 promoter could provide an initial way forward with functional studies in less well studied species, for example the helminth parasites of humanity at large, until endogenous (congruent) U6 genes could be isolated and tested. In relation to *S. mansoni* or other schistosome species, deploying the human U6 gene promoter should allow studies to progress while other copies of the schistosome U6 gene can be investigated.

To conclude, these findings indicated superior performance of a human U6 promoter over a schistosome U6 promoter in cassettes driving shRNAs to silence reporter genes in schistosomes, at least in blood stage schistosomules of *S. mansoni*. We are interested to establish long-term, stable and trans-generational RNAi to facilitate investigation of the essentiality of schistosome genes which could, in turn, be targeted in new approaches for treatment and control of schistosomiasis. The human U6 gene promoter clearly represents a potentially valuable transgene element for shRNA studies in schistosomes. High throughput, vector-based RNAi analysis can be expected to advance functional genomics for schistosomes in assisting the discovery of novel targetable genes (Stefanic et al. 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic illustration of the retroviral pLNHX (murine leukemia virus, MLV) and pXL-Bac II (transposon *piggyBac*) based plasmids (left) and shRNA expression cassettes (right) modified here by insertion of shRNA expression cassettes. The shRNA expression cassettes target firefly luciferase or a 'scrambled' control sequence. MCS, multiple cloning site; ITR, inverted terminal repeat (regions of the transposon); LTR, long terminal repeat (of the retrovirus); cHS4, chicken DNase-I hypersensitive site 4 (a prototypic chromatin insulator).

Figure 2.

Human U6 gene promoter driving short hairpin RNA (shRNA) in human HT1080 fibrosarcoma cells and seven day old schistosomules of *Schistosoma mansoni* after transduction with MLV retroviral virions. **Panel A:** Knockdown of luciferase (47% knockdown) in HT1080 cells transduced with pLNHX_HsU6_shLuc (HsU6 shLuc) virions compared to control (HsU6 Scram) virions. **Panel B:** Knockdown (80%) of luciferase in schistosomules transduced with pLNHX_HsU6_shLuc (HsU6 shLuc) virions compared to control (HsU6 Scram) virions. Luciferase activity is expressed as relative light units per second per microgram of soluble protein (RLU/s/μg).

Figure 3.

Comparison between human U6 and schistosome U6 promoters driving short hairpin RNA targeting firefly luciferase in seven day old schistosomules after transduction with MLV retroviral virions. Transduction with the human U6 promoter based pLNHX_HsU6_shLuc virions lead to knockdown (38%) whereas no knockdown was evident after transduction with the schistosome U6 promoter based pLNHX_SmU6_shLuc virions, when compared to the two control groups. These two control schistosomules were transduced with control MLV (pLNHXΔ) virions and not transduced with virions (mock) Luciferase activity is expressed as relative light units (RLU) per second per microgram of soluble protein (RLU/s/μg).

Figure 4.

Comparison between human U6 and schistosome U6 promoter driving short hairpin RNAs targeting firefly luciferase in a HT1080 cell line stably expressing firefly luciferase and in 14 day old schistosomules of *Schistosoma mansoni* after transfection with pXL-Bac II plasmids. **Panel A:** Strong knockdown was seen in cells transfected with both the human U6 (88% knockdown) and schistosome U6 (75%) promoters in comparison with mock-treatment control. By contrast, much less knockdown was apparent in controls transfected with the human U6 and schistosome scrambled target sequence based plasmids, 30 and 29% respectively.

Panel B: In schistosomules, much stronger knockdown was seen with human U6 promoter (80% knockdown) compared with schistosome U6 promoter (30% knockdown) driving shRNA after transfection with pXL-Bac II plasmid constructs. Minimal or no knockdown was seen with the control plasmids, 15 and 0%. In both panels A and B, luciferase activity is expressed as relative light units per second per microgram of soluble protein (RLU/s/μg).