Using RNA interference to manipulate endogenous gene expression in Schistosoma mansoni sporocysts

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Received 2 September 2002; received in revised form 3 March 2003; accepted 17 March 2003

Abstract

Direct assessments of gene function in parasitic flatworms have been hampered by the lack of effective tools to alter gene expression. The aim of the present study was to use RNA-interference (RNAi) to achieve targeted gene knockdown in larval stages of the human blood fluke, Schistosoma mansoni. We selected two S. mansoni genes for RNAi experiments: SGTP1, a facilitated diffusion glucose transporter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). When S. mansoni larvae were treated in vitro for 6 days with dsRNA specific to one of these two genes, targeted transcript levels were reduced by 70–80% as determined by quantitative PCR (qPCR), while non-targeted transcripts were unaffected. Parasite exposure to SGTP1 dsRNA, but not GAPDH dsRNA, reduced larval glucose-uptake capacity by 40%, demonstrating that SGTP1 transcript knockdown results in the functional phenotype of reduced glucose transport activity. The effect of dsRNA treatment on transcript level was evident for up to 28 days after an initial dsRNA treatment. Interestingly, dsRNA treatment was effective only when miracidia were allowed to undergo the transition to sporocysts in its presence, while treatment of fully transformed sporocysts was ineffective. Fluorescence patterns in larvae exposed to rhodamine-labeled dsRNA as miracidia and sporocysts were similar, suggesting that the difference in susceptibility to dsRNA treatment between the two life stages may not be due to differences in dsRNA entry. Overall, this technology will enable direct assessment of the roles of individual genes in physiological processes of larval stages of S. mansoni, a crucial step in the identification of novel intervention targets for this important human pathogen.

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Keywords: Schistosoma mansoni; Sporocyst; RNA interference; Quantitative PCR

1. Introduction

RNA-interference (RNAi) is a process by which the introduction of exogenous double-stranded RNA corresponding to a specific messenger RNA sequence results in a significant reduction in levels of the targeted mRNA, and in phenotypes that are similar to those achieved using gene-knockout or mutation technologies [1]. Originally developed for use in Caenorhabditis elegans [2], it has been shown to be effective in a wide variety of organisms, including protozoans [3,4], planarians [5,6], parasitic nematodes [7], Drosophila melanogaster [8], and mammals [9,10], and is thought to occur via dsRNA-mediated degradation of the corresponding transcript [12,11]. RNAi has many advantages over more conventional methods of altering gene expression. As long as a portion of a particular mRNA sequence is known, the effects of altering its expression can be assessed by introducing dsRNA corresponding to the desired sequence, circumventing the need for the generation and selection of stable genetic transformants containing DNA insertions of interest. However, for RNAi to be useful in a particular organism: (1) there must be an effective method to deliver the exogenous dsRNA, (2) the organism or tissues targeted must express the protein machinery necessary to process the dsRNA and to direct degradation of the targeted transcript [11–13], and (3) the presence of dsRNA itself should not result in either the degradation of non-targeted transcripts [14] or in the induction of stress responses in the target tissue [9,15].

Schistosoma mansoni is one of four major Schistosoma spp. that infect over 200 million humans in over 70 countries [16,17], and its importance as a human health problem...
in the tropics has led to substantial research interest in this organism. Recent years have witnessed a dramatic increase in the number of identified genes from *S. mansoni*, resulting from both expressed sequence tag (EST) [18] and genomic survey projects [19]. However, despite recent progress in the transient transfection of different stages of *S. mansoni* with reporter constructs [20–21], direct analyses of gene function have been hampered by the lack of effective tools for genetic manipulation. We therefore tested whether dsRNA could be used to interfere with gene expression in the first intramolluscan larval stage of *S. mansoni*, the mother sporocyst. This particular life stage can be easily maintained in the laboratory [22], and the entire molluscan life cycle can be propagated in vitro [23–25] making it a unique and tractable system to test the utility of RNAi to assess gene function in *S. mansoni*. Our results demonstrate that properly-timed treatment of developing larvae with sequence-specific dsRNA results in significant reduction of targeted transcript levels. This represents the first example of targeted gene knockdown in any species of parasitic flatworm and it is anticipated that this technique will prove to be useful to address gene function not only in larvae of *S. mansoni*, but also in other trematode species of medical or agricultural importance [22].

2. Materials and methods

2.1. Parasites

The NMRI strain of *S. mansoni* was used in all experiments. Miracidia were hatched in artificial pond water from eggs recovered from homogenized livers of 7–8 weeks infected mice [26]. Free-swimming miracidia were washed and pelleted by incubation on ice and centrifugation at 26°C (700 g), and pelleted by incubation on ice and centrifugation at 26°C (700 g) and 10,000 g for 10 min. Pellets were resuspended in 26°C (700 g) and 10,000 g for 10 min. Pellets were resuspended in CBSS (pH 7.2; NaCl (47.9 mM), KCl (3.6 mM), CaCl₂ (0.6 mM), NaHCO₃ (0.6 mM)) [27] supplemented with 2% fetal bovine serum (Sigma, St. Louis, MO) and antibiotics as described [24].

2.2. B. glabrata embryonic (Bge) cells/long-term sporocyst cultures

Bge cells have been used extensively for the long-term in vitro culture of intramolluscan stages of *S. mansoni* [22], and details of the culture methods used in this study have been described previously [22,24]. Cells were routinely maintained at 26°C in Bge medium [28] supplemented with heat-inactivated 10% fetal bovine serum (Sigma, St. Louis, MO). Culture medium was exchanged every 3–5 days.

2.3. Targeted genes

We selected two *S. mansoni* genes for interference studies: an *S. mansoni* facilitated-diffusion glucose transporter (SGTP1; GenBank™ Acc. No. L25065) [29] and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank™ Acc. No. M92359) [30]. These genes were chosen because their functions have been well-characterized in heterologous expression systems [29,30], and preliminary analysis using quantitative PCR (qPCR) in our laboratory revealed that they were expressed in sporocyst stages of *S. mansoni* (data not shown). We therefore tested whether dsRNA could be used to interfere with gene expression in sporocysts of *S. mansoni* (data not shown).

2.4. dsRNA synthesis

Double-stranded RNA molecules were synthesized using “double-barreled” DNA templates engineered to contain the T7 RNA polymerase promoter sequence at each end as described by Clemens et al. [8]. Gene-specific primers were used to PCR amplify either a 448 (SGTP1) or a 452 (GAPDH) bp fragment directly from sporocyst cDNA using the following primers: (5′–3′): SGTP1: forward gcttcttctataaatgccgta, reverse gcttgctgccgctggtc; reverse gcttgctgccgctggtc (corresponding to nucleotides 193–640 of the complete mRNA sequence); GAPDH: forward gtgttttatcataaagctcag, reverse gatgttgattgctggtc (corresponding to nucleotides 439–891 of the complete mRNA sequence). Similar primers with the T7 RNA polymerase sequence added to the 5′ end were used in a subsequent PCR reaction using the gel-purified primary PCR product as template. dsRNA was synthesized from these templates using the MegaScript™ T7 RNA polymerase kit (Ambion, Austin, TX) according to the manufacturer’s instructions, except that reactions were allowed to proceed overnight (10–16 h) to increase RNA yield. RNA was DNAse treated, extracted with phenol:chloroform, and isopropanol-precipitated pellets were dissolved in diethylylcarbamate (DEPC)-treated water and stored at −20°C. dsRNA was used within 24 h of synthesis and its integrity was routinely verified by non-denaturing agarose gel electrophoresis.

2.5. dsRNA treatments

Following a final wash in sterile pond water, miracidial pellets were resuspended in CBSS [28] and quantified. Approximately 5000 miracidia were added to individual wells of 48-well plates in 250 μl CBSS [28] and 250 μl of a 2× concentration of the required amount of dsRNA in CBSS [28], or CBSS alone was added directly to wells and
mixed by pipetting. These manipulations were carried out within 10 min of exposure of the miracidia to CBSS\(^\text{+}\), and in all experiments plates were incubated at 26 °C for 6 days prior to further manipulations. Sporocysts were then washed free of dsRNA with CBSS, and either harvested immediately for transcript quantification or glucose uptake assays, or maintained in culture for further manipulation. In preliminary tests of RNAi, SGTP1 and GAPDH dsRNA concentrations ranged from 1.0 to 100 nM. To examine changes in \(S.\ mansoni\) susceptibility to dsRNA treatment during the miracidium-to-sporocyst transformation process, 50 nM SGTP1 dsRNA was added to culture wells at the following times after culture initiation: after 10 min, 1, 2, 6, and 24 h.

2.6. Longevity of RNAi effect on targeted transcript levels in cultured sporocysts

To determine the longevity of the effect of dsRNA on transcript levels, we exploited the ability to carry out long-term in vitro culture of \(S.\ mansoni\) sporocysts in the presence of Bge cells or their secreted products [24]. Depending on the duration of the cultivation period, sporocysts were maintained under different culture conditions after being treated with dsRNA for 6 days and washed. To assess the degree of interference 14 days post-dsRNA treatment, sporocysts were harvested for qPCR (see below) to assess the degree of specific interference. To better assess the long-term effects and specificity of RNAi on transcript levels, in addition to determining SGTP1 and GAPDH transcript levels, we quantified transcripts for three other \(S.\ mansoni\) genes: a RAS homologue (GenBank\textsuperscript{TM} Acc. No. U53177) [32], phosphofructokinase (PFK; GenBank\textsuperscript{TM} Acc. No. L31531) [33], and a neurotransmitter transporter that is not dependent on extracellular Na\(^+\) transport [29]. For each primer/cDNA sample combination, reactions were carried out in duplicate. qPCR reaction mixtures contained 200 U of MMLV-RT (Promega, Madison, WI) according to the manufacturer’s instructions.

qPCR was carried out on an Applied Biosystems 5700 PCR apparatus using SYBR\textsuperscript{®} green dye (Molecular Probes, Eugene, OR) to quantify product formed over the course of the PCR reaction [34]. qPCR primers were designed using Primer Express\textsuperscript{®} Software (Applied Biosystems, Foster City, CA) and were selected if they had melting temperatures between 58 and 60 °C, would produce an amplicon in the range of 50–150 bp, and only if they had two or fewer G–C pairs in the final 5 bp of the 3' end. SGTP1 qPCR primers were located downstream (3') of the targeted region by the dsRNA, while those for GAPDH were located upstream (5') of the targeted region. Primer pairs used for qPCR were as follows (target gene: forward, reverse [region amplified]): SGTP1: gaagcagtcgctcatggtggag, aagctgtagctgctctcaaca [1410–1470]; GAPDH: tcttgagatctctggagtttgc, aataagcgtctgcttacattg [284–336]; RAS: caagaagagttggaagaatc, tgttgctggctttcatct [452–552]; PFK: gatcggcgtccagct, atctccttgattctcgttataacc [1388–1488]; and SmNT: agggcgcgcgccggat, caattttgctgttccgagac [1245–1295].

Complementary DNA samples were diluted 1:3 with sterile H\(_2\)O, and either used directly in qPCR reactions (for poly-adenylated RNAs), or diluted 100-fold further with H\(_2\)O for quantification of 18s ribosomal transcript levels. For each primer/cDNA sample combination, reactions were carried out in duplicate. qPCR reaction mixtures contained the following: 2.5 μl diluted cDNA, 240 nM of each primer, 200 μM each of dATP, dCTP, and dGTP, 225 μM ROX-I dye labeled 6-mer (Synthegen, Houston, TX) in low Na\(^+\) CBSS. Each assay had a total volume of 20 μL, and the final d[6-3H]glucose concentration was 2 μM (60 μCi ml\(^{-1}\)). After 10 min, assays were terminated by two 1.5 ml washes in ice-cold low Na\(^+\) CBSS. Sporocysts were then resuspended in 100 μl CBSS, and 85 μl of the suspension was added to 3.5 ml Scintiverse\textsuperscript{®} scintillation fluid (Fischer) and subjected to liquid scintillation counting. The number of sporocysts in 10 μl of the suspension were counted to determine uptake per 1000 sporocysts. Non-specific uptake was determined by carrying out identical assays in the presence of 10 mM unlabeled glucose at 4 °C. Assays were performed in triplicate for each group of sporocysts and averaged prior to analysis.

2.8. RNA harvesting/quantitative PCR

Total RNA was harvested from sporocysts using the RNAzol\textsuperscript{TM} (Tel-Test, Friendswood, TX) reagent according to the manufacturer’s instructions. Isopropanol-precipitated pellets were dissolved in DEPC-treated water, and 2 μg of total RNA were reverse-transcribed in a 25 μl reaction containing 200 U of MMLV-RT (Promega, Madison, WI) according to the manufacturer’s instructions. 

For each primer/cDNA sample combination, reactions were carried out in duplicate. qPCR reaction mixtures contained the following: 2.5 μl diluted cDNA, 240 nM of each primer, 200 μM each of dATP, dCTP, and dGTP, 225 μM ROX-I dye labeled 6-mer (Synthegen, Houston, TX) mixed by pipetting. These manipulations were carried out within 10 min of exposure of the miracidia to CBSS\(^\text{+}\), and in all experiments plates were incubated at 26 °C for 6 days prior to further manipulations. Sporocysts were then washed free of dsRNA with CBSS, and either harvested immediately for transcript quantification or glucose uptake assays, or maintained in culture for further manipulation. In preliminary tests of RNAi, SGTP1 and GAPDH dsRNA concentrations ranged from 1.0 to 100 nM. To examine changes in \(S.\ mansoni\) susceptibility to dsRNA treatment during the miracidium-to-sporocyst transformation process, 50 nM SGTP1 dsRNA was added to culture wells at the following times after culture initiation: after 10 min, 1, 2, 6, and 24 h.

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Complementary DNA samples were diluted 1:3 with sterile H\(_2\)O, and either used directly in qPCR reactions (for poly-adenylated RNAs), or diluted 100-fold further with H\(_2\)O for quantification of 18s ribosomal transcript levels. For each primer/cDNA sample combination, reactions were carried out in duplicate. qPCR reaction mixtures contained the following: 2.5 μl diluted cDNA, 240 nM of each primer, 200 μM each of dATP, dCTP, and dGTP, 225 μM ROX-I dye labeled 6-mer (Synthegen, Houston, TX) in low Na\(^+\) CBSS. Each assay had a total volume of 20 μL, and the final d[6-3H]glucose concentration was 2 μM (60 μCi ml\(^{-1}\)). After 10 min, assays were terminated by two 1.5 ml washes in ice-cold low Na\(^+\) CBSS. Sporocysts were then resuspended in 100 μl CBSS, and 85 μl of the suspension was added to 3.5 ml Scintiverse\textsuperscript{®} scintillation fluid (Fischer) and subjected to liquid scintillation counting. The number of sporocysts in 10 μl of the suspension were counted to determine uptake per 1000 sporocysts. Non-specific uptake was determined by carrying out identical assays in the presence of 10 mM unlabeled glucose at 4 °C. Assays were performed in triplicate for each group of sporocysts and averaged prior to analysis.
TX), 0.1× SYBR green, and 0.2 U of HotStar Taq polymerase (Qiagen, Valencia, CA). Reactions were cycled as follows on a GeneAmp® 5700 Sequence Detection system (Applied Biosystems): 95 °C for 15 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Dissociation curves were carried out at the end of each run to determine product purity. In general, assays for each sample consisted of three primer sets: one for the gene being interfered with (target gene), one for a gene not being interfered with (non-target specificity control), and one for 18s ribosomal transcripts to serve as a loading control (Sm18s: forward, ggctggccacggatct; reverse, ccggctacgaccctgatt [299–420]; GenBank™ Acc. No. U65657). qPCR data were analyzed with GeneAmp® 5700 SDS software (Applied Biosystems). Typically the amplification threshold was set at 0.2, and the background fluorescence was determined during cycles 6–10. In cases where these values were adjusted, they were always set at the same value for each primer set.

2.9. Rhodamine-labeled dsRNA

To determine the efficiency of dsRNA uptake in *S. mansoni* larvae of different ages, 5 μg of SGTP1 dsRNA was labeled with 5-carboxy-X-rhodamine using the Label It® Nucleic Acid Labeling Reagent (Mirus Corporation, Madison, WI) according to the manufacturer’s instructions. Labeled dsRNA was purified by ethanol precipitation, and the pellet was washed 2 × with 1.5 ml of 70% ethanol to remove any excess rhodamine present in the supernatant. Direct labeling of the RNA was confirmed by running an aliquot of the ethanol-purified, labeled dsRNA on an agarose gel and visualizing the correctly-sized fluorescent band on an UV box without staining the gel with ethidium bromide. Freshly hatched *S. mansoni* miracidia were plated in CBSS™ at a density of 1000 per well in 96-well plates, and larvae were treated with 100 nM rhodamine-labeled dsRNA either within 10 min of plating (miracidia treatment), or after 20 h in culture (sporocyst treatment). In both cases incubation with labeled dsRNA was allowed to proceed for 4 h, larvae were washed 2 × with 1.5 ml CBSS™, and then fixed for 30 min in 4% paraformaldehyde in saline phosphate-buffered saline (pPBS) [24]. After washing 4 × 1.5 ml with pPBS, larvae were placed on glass coverslips and visualized by either standard fluorescence microscopy or confocal microscopy. Miracidia and sporocysts that were not treated with labeled dsRNA served as controls, and images were manually adjusted for contrast and brightness in a uniform fashion for qualitative analysis and presentation using Adobe Photoshop®.

2.10. Statistical analyses

Statistical analysis software (SAS, Cary, NC) was used to analyze all data using the General Linear Models procedure and a randomized complete block design with experimental replicate as the blocking factor [35]. Multiple means post-tests were made using either the Least Squared Means procedure (for comparing each experimental treatment to the control value), or using Fischer’s Least Significant Difference test (for comparing each value to all other values) in SAS.

For graphical representation of qPCR data, raw cycle threshold (Ct) values obtained for each sample were converted to fraction of control transcript levels using the delta-delta Ct (ΔΔCt) method [36], with 18s ribosomal gene levels serving as the internal standard. In order to make statistical comparisons of treatment groups, it was not possible to analyze data transformed using the ΔΔCt method since it had been converted to fraction of the reference sample. Instead, the Ct value of the 18s ribosomal gene was subtracted from the Ct value of the gene of interest for each sample/primer combination, and these raw values (ΔCt) were analyzed using ANOVA and multiple means comparisons. These particular methods are summarized in Table 1.

3. Results

3.1. Specificity of dsRNA-interference and effects of SGTP1 dsRNA on glucose uptake

Our first goal was to determine whether direct treatment of *S. mansoni* larvae with dsRNA would specifically reduce targeted transcript levels. When fully transformed sporocysts were cultured for 6 days in the presence of dsRNA for SGTP1, no significant reductions in targeted transcript levels were detected (data not shown). However, when miracidia were allowed to transform in the presence of either 50 nM SGTP1 or GAPDH dsRNA and then cultured for 6 days, we detected significant reductions in target transcript levels ranging from 60 to 90%, and this dsRNA treatment did not significantly alter levels of transcripts that were not targeted by the dsRNA. As shown in Fig. 1A, SGTP1 levels in sporocysts treated as miracidia with 50 nM SGTP1 dsRNA and cultured for 6 days were reduced to 18 ± 0.14% of control levels ($P = 0.0001$), while GAPDH levels in these same sporocysts were unaffected ($P = 0.9880$). The same held true for sporocysts treated with GAPDH dsRNA, in that GAPDH levels were reduced to 28 ± 0.12% of control

| Table 1: Method for data normalization and statistical analysis of qPCR data.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔCt SGTP1</th>
<th>ΔCt 18s</th>
<th>ΔΔCt</th>
<th>ΔΔCt/delta1</th>
<th>2$^{-\Delta\Delta Ct}$</th>
</tr>
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<td>Control</td>
<td>21.92</td>
<td>16.64</td>
<td>5.28</td>
<td>0.00</td>
<td>1.00</td>
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<td>23.81</td>
<td>16.32</td>
<td>7.49</td>
<td>2.22</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Fig. 1. Specificity of dsRNA effects on levels of targeted transcripts (A) and glucose uptake (B) in
S. mansoni larvae. Freshly hatched miracidia were treated with either 50 nM SGTP1 or GAPDH dsRNA, or carrier alone (control), and cultured for 6 days. Data are represented as fraction of control ± the standard error of the mean (S.E.M.) for graphical representation only. **P<0.0001 and *P<0.01 when compared to controls as determined by ANOVA and Least Squared Means post tests.

N= five/treatment. (A) Relative levels of SGTP1 (black bars) and GAPDH (white bars) transcripts were determined using quantitative PCR as described in Section 2. The presence of either SGTP1 or GAPDH dsRNA resulted in the significant reduction in levels of the corresponding transcript, while the non-targeted gene was not affected. (B) Sporocysts were washed extensively in low Na+ medium, and subjected to a tritiated glucose uptake assay. Sporocysts treated with SGTP1, but not GAPDH, dsRNA exhibited significantly reduced Na+—independent glucose uptake levels (P<0.0001), while SGTP1 levels were not significantly different from controls (P = 0.2045) (Fig. 1A).

While dsRNA treatment is presumed to act directly on nascent transcripts, we also wanted to determine its effects on levels of functional protein. The S. mansoni SGTP1 gene is known to confer Na+—independent glucose uptake capacity when expressed in Xenopus oocytes [29], and therefore we assessed Na+—independent glucose uptake in dsRNA-treated sporocysts. As shown in Fig. 1B, sporocysts treated with 50nM SGTP1 dsRNA for 6 days had glucose uptake capacity that was 61 ± 6.0% of that observed in untreated controls (P = 0.0015), while glucose uptake capacity in sporocysts treated with GAPDH dsRNA was unaffected (88 ± 8.2% of untreated controls; P = 0.2455). This significant reduction in Na+—independent glucose uptake in SGTP1 dsRNA-treated sporocysts suggests that reductions in transcript level caused by dsRNA treatment result in decreases in the corresponding protein product.

When sporocysts were treated with different concentrations of SGTP1 dsRNA, SGTP1 transcript levels were significantly reduced by treatment with concentrations as low as 10nM (69 ± 22% of control; P = 0.0093) (Fig. 2A). When GAPDH levels were assessed in these same sporocysts, no non-specific decreases in transcripts for this gene were observed at any of the concentrations tested (P > 0.05; data not shown). When glucose uptake capacity was assessed in similarly-treated sporocysts, Na+—independent glucose uptake was significantly reduced in sporocysts treated with both 100 and 50nM SGTP1 dsRNA (P < 0.05) (Fig. 2B). While uptake capacity of 10nM dsRNA-treated sporocysts was 72 ± 8.1% of control, this was not significantly lower than control uptake (P = 0.0936). However, this general

![Fig. 2](image-url)
correlation between treatments that reduced SGTP1 transcript levels and those that reduce Na\(^+\)-independent glucose uptake capacity implies that presumed dsRNA-mediated degradation of specific transcripts results in a functional phenotype; in this case the ultimate disruption of physiological processes carried out by the corresponding encoded protein.

3.2. Effect of sporocyst age on susceptibility to dsRNA

As described above, dsRNA treatments were only effective if larvae were placed in its presence immediately after initiation of in vitro cultures. We therefore examined the effect of adding 50 nM SGTP1 dsRNA at different times after culture initiation. As shown in Fig. 3A, as the time between culture initiation and dsRNA treatment increased, the effectiveness of the dsRNA treatment, as measured by levels of the targeted transcript (SGTP1), decreased. dsRNA was most effective when added either immediately (within 10 min) or 1 h after initiation of cultures, where SGTP1 levels were 15 ± 8.3 and 17 ± 15% of control, respectively. When dsRNA was added either 2 or 6 h after culture initiation, SGTP1 levels were reduced compared to controls, but these levels were both significantly higher (P < 0.05) when compared to those resulting from immediate treatment (Fig. 3A). When SGTP1 dsRNA was added 24 h after culture initiation, SGTP1 transcript levels were very near untreated control values (72 ± 16% of control), and not significantly different (P > 0.05). These changes in susceptibility to dsRNA correlated with morphological changes observed in larvae at these same time points, as shown in Fig. 3B and D. Almost immediately (within 10 min) after culture initiation (CBSS\(^+\) addition), free-swimming miracidia began to settle on the bottom of the culture well, and by 60 min many of the settled larvae were round in shape (Fig. 3B). By 2 h, dramatic changes in morphology and behavior were evident. Over 60% of the miracidia ceased swimming behavior, and most had a rounded shape. In many of these larvae, individual ciliary epidermal plates were observed detaching from the larval surface, although very few plates had been cast off by this point (Fig. 3C). By 6 h, ~50% of the larvae had completely shed their ciliary plates and possessed a fully-formed tegument (Fig. 3D), completing the miracidium-to-sporocyst transformation process, and over 75% of larvae had fully transformed into sporocysts after 24 h in culture. These data suggest that there is a “window of opportunity” shortly after the initiation of miracidial cultures during which dsRNA is effective, and that as the

Fig. 3. (A) Effect of sporocyst age on efficiency of SGTP1 dsRNA treatment. Miracidia were cultured in CBSS\(^+\) for the times indicated prior to the addition of 50 nM SGTP1 dsRNA. After 6 days in culture, sporocysts were harvested and SGTP1 levels were determined using quantitative PCR as described in Section 2. Treatment within either 10 min or 1 h of culture initiation resulted in the highest reduction in SGTP1 transcript levels, while dsRNA treatment at later times was less effective as determined by ANOVA and Fischer’s Least Significant Difference test. Means with the same letter are not significantly different (P > 0.05). N = three/treatment. (B–D) Photomicrographs of morphological changes in miracidia at different times after culture initiation. Immediately after culture initiation (B) free-swimming miracidia began to exhibit a rounded shape (arrowhead) and settle on the bottom of the culture dish (200× magnification). By 2 h post-culture-initiation (C), ciliary plates (arrowhead) were observed to be in the process of detaching from developing larvae (400×), and after 6 h in culture (D) many plates (arrowheads) had been fully shed from the larvae, thereby completing the process of miracidium-to-sporocyst transformation (400×).
Fig. 4. Fluorescence patterns in larvae treated with rhodamine-labeled dsRNA as freshly-hatched miracidia (A–D, J) or as 20 h sporocysts (E–H, K).

(A–D) Immediately after hatching, *S. mansoni* miracidia were treated with 100 nM rhodamine-labeled dsRNA in CBSS+ for 4 h, and then fixed, washed, and viewed using light (A, C) and fluorescent (B, D) microscopy. Very little fluorescent signal was detected in larvae that had yet to transform into sporocysts (i.e. miracidia), barring some staining on the apical end of the miracidium (arrow, B). In certain cases, larvae that were in the process of transforming into sporocysts (C, D) contained fluorescent signal most likely within the body of the worm (arrow, D). (E–H) *S. mansoni* miracidia were placed into culture in CBSS+ for 20 h to induce transformation into sporocysts, and then treated for 4 h with 100 nM labeled dsRNA and processed for microscopy as above. In certain cases, fluorescent signal was observed in or associated with the surface tegument in sporocysts (F), while in other cases dispersed, possibly internal, staining was observed (arrowheads, H). (I–K) Similarly-treated *S. mansoni* larvae were examined using confocal microscopy. (I) Background fluorescence (in the absence of any rhodamine-labeled dsRNA). (J) Optical section of larvae treated with dsRNA as a freshly-hatched miracidium, showing example of cell-localized fluorescent signal (arrow). (K) Optical section showing similar staining in a larva treated with labeled RNA after 20 h in culture (arrow). Magnification: A–H (400×), I–K (1000×).

The larval transformation process occurs the developing sporocysts become refractory to its effects on transcript levels.

3.3. Effect of sporocyst age on entry of rhodamine-labeled dsRNA

To investigate the possibility that dsRNA treatments were not effective in fully transformed sporocysts due to lack of efficient entry of exogenous RNA, we compared fluorescence patterns in miracidia and 20 h sporocysts exposed to rhodamine-labeled dsRNA. When miracidia were treated immediately after culture initiation with labeled dsRNA, the fluorescence pattern observed was dependent on the degree to which an individual larvae had undergone the transformation process. In those larvae that did not transform into sporocysts during the 4 h incubation, we observed very little fluorescent signal above background, barring small areas of punctate staining at the anterior (arrow, Fig. 4B) and the posterior (not shown) end of the larvae. In contrast, some larvae that had begun or completed the transformation process were highly fluorescent, with staining present in or associated with the surface tegument (not shown) and other structures within the body of the larvae (arrow, Fig. 4D). However, not all larvae in this treatment group that were undergoing the transformation process were fluorescent. An example of this is shown in Fig. 4C and D: the larvae on the left side of the micrograph clearly began the transformation process prior to fixation (as evidenced by missing ciliary plates) (Fig. 4C), but very little fluorescent signal was observed.

When larvae that had been in culture for 20 h were treated with labeled dsRNA for 4 h, similar patterns of fluorescence were observed. In those cases where miracidia had yet to transform into sporocysts, little or no fluorescent staining was evident (not shown). Some larvae that had transformed into sporocysts prior to the addition of the labeled dsRNA...
displayed intense peripheral fluorescence (Fig. 4F), which may merely represent tegumental surface staining, and not actual entry of the fluorescent label. However, other larvae displayed clear staining in internal worm structures (arrows, Fig. 4H). Again, not all sporocysts were fluorescent, as exemplified by the larvae at the top of the micrograph in Fig. 4F. Qualitatively, both the fluorescence intensity and the fraction of larvae (excluding non-transformed larvae, i.e. miracidia) that were detectably fluorescent did not differ between those treated with labeled dsRNA immediately after hatching and those treated after 20 h in culture.

To better determine the fate of exogenous labeled dsRNA in these experiments, larvae were also examined using confocal microscopy. Using this method, it was not possible to determine whether the peripheral fluorescence observed in sporocysts (shown in Fig. 4F) was actually present within the worm tegumental syncytium, or if it was bound to the outer surface of the worm, and therefore we focused on localizing the fluorescent signal in those larvae with more dispersed staining (as in Fig. 4D and H). Regardless of the treatment group examined, optical sectioning revealed that fluorescent label was clearly within the body of the larva, and in certain cases was present within specific cells (see arrows, Fig. 4J and K). While these cells appear to be similar in shape and size to germinall cells [37], it was difficult to determine their identity at the magnification used (1000×).

3.4. Longevity of dsRNA-interference

Using two different methods of long-term culture of S. mansoni sporocysts treated with dsRNA, we determined the duration of the effect of SGTP1 RNAi. As shown in Fig. 5, the effects of SGTP1 dsRNA treatment persists at both 14 and 28 days after dsRNA washout. At 14 days post-wash, SGTP1 levels in SGTP1-treated sporocysts were 18 ± 6.7% of control (P = 0.0139) (Fig. 5A). After 28 days, SGTP1 levels were still significantly lower than controls, although the degree of interference was reduced compared to 6 and 20 days samples (60 ± 7.9% of control; P = 0.008) (Fig. 5B). In contrast to the 6 day treatment, we did observe decreases in levels of the non-targeted gene GAPDH in SGTP1-treated sporocysts 14 days post-wash (60 ± 13% of control) (Fig. 5A). While these reduced levels were not statistically significant (P > 0.15), they were consistent in our 14 days samples. To determine if this was due to promiscuous degradation of non-targeted transcripts, in the same qPCR runs we quantified levels of three other transcripts for both 14 and 28 day samples: RAS, phosphofructokinase (PFK), and a neurotransmitter transporter homologue currently under investigation in our laboratory (SmNT). At 14 days post-wash, PFK and RAS levels also were depressed in SGTP1 dsRNA-treated sporocysts relative to controls (72 ± 7.9% and 76 ± 5.8% of control, respectively), while SmNT levels were very near control values (98 ± 17% of control). The differences in mRNA levels for these non-targeted genes in the 14 days samples were not statistically significant (P > 0.15) (Fig. 5A), suggesting that the changes in gene levels we observed is not due to non-specific dsRNA-mediated degradation of non-targeted transcripts [14]. In 28 day samples, levels of the four non-targeted transcripts were indistinguishable from controls (P > 0.15) (Fig. 5B).

4. Discussion

In the present study we report that dsRNA treatment results in the disruption of the expression of endogenous genes in the intramolluscan sporocyst stage of the human blood...
fluke, S. mansoni. To our knowledge, this is the first report of targeted manipulation of endogenous gene expression in a parasitic flatworm. Using qPCR with SYBR® green detection, we observed significant reductions (~80%) in levels of two different S. mansoni genes after treatment with the corresponding dsRNA, but did not detect reductions in levels of non-targeted transcripts. While we have not found other reports in the literature using qPCR to assess transcript level reductions resulting from RNAi, the percent reductions that we observe in S. mansoni larvae are similar to those obtained in other organisms using alternate methods to quantify transcripts. For example, in Trypanosoma brucei Northern blotting was used to assess alpha- and beta-tubulin levels after introduction of the corresponding dsRNA, and transcript level reductions ranged from 50 to 85% [8]. In mouse oocytes, semi-quantitative RT-PCR demonstrated that dsRNA treatment reduced transcript levels by 80–90% [10], and similar numbers were obtained in C. elegans [38]. While it is clear from the present study and others [2] that dsRNA treatment does not result in the complete elimination of the corresponding message, in many documented cases the level of transcript degradation is sufficient to generate phenotypes expected from reduced expression of the corresponding gene [39].

Schistosomes are known to express a wide variety of solute transporters on their tegumental surface, facilitating the uptake of a wide variety of molecules from the host environment [40]. SGTP1 is a facilitated-diffusion glucose transporter that is expressed at the basal lipid bilayer of both adult and larval stages of S. mansoni [31] and Skelly PJ and Shoemaker CB, unpublished observations), where it is likely to mediate Na⁺-independent uptake of glucose from the tegumental cytoplasm into the body of the worm. When we examined uptake of exogenous glucose in SGTP1 dsRNA-treated larvae, we observed significant (~40%) reductions in Na⁺-independent glucose transport that were highly correlated with treatments that resulted in reduced transcript levels. Reduced uptake was not due to a non-specific effect of dsRNA treatment since GAPDH dsRNA had no effect on exogenous glucose transport. Thus, the data suggest that SGTP1 is indeed expressed on one (or both) of the bilayers on the tegumental surface, and provides further evidence that specific dsRNA-mediated reduction of the targeted transcript results in lower levels of the corresponding protein product. However, SGTP1 treatment did not completely eliminate Na⁺-independent glucose uptake, implying that other facilitated diffusion glucose transporters are present [29], and/or that turnover of pre-formed transporter proteins is a slow process. In addition, there is very little similarity at the nucleic acid level between SGTP1 and other glucose transporters cloned from S. mansoni (SGTP2; GenBank® Accession No. L25066; SGTP4; GenBank® Accession No. L25067) [29], and therefore SGTP1 dsRNA treatment would not be expected to disrupt expression of these non-targeted genes. This is in contrast to recent studies using RNAi in the parasitic nematode Nipponstrongylus brasiliensis, where there appeared to be enough sequence similarity between secreted acetylcholinesterases (67–90% in the targeted region) such that treatment with dsRNA corresponding to one (ACHE B) resulted in a global reduction in secreted ACHe activity due to disruption of at least two other mRNAs [7].

Despite the significant reductions observed in activities associated with SGTP1, larvae treated in this manner demonstrated no significant morphological or behavioral variations from controls. The same held true for sporocysts treated with dsRNA specific for the glycolytic enzyme GAPDH. While this may seem troubling, there are many genes for which RNAi gives no detectable changes in morphology, behavior, or viability. For example, when C. elegans was subjected to RNAi for all genes of chromosome I, only 14% of the tested genes gave observable phenotypes [41], and there was a similar lack of clear phenotype when RNAi was used to interfere with acetylcholinesterase in the nematode N. brasiliensis [7]. In addition, schistosomes exhibit significant developmental plasticity, and are able to switch between metabolic pathways in response to changes in the environment [42]. This metabolic plasticity, coupled with possible compensation by redundant genes, is a reasonable explanation as to why sporocysts with dramatically reduced levels of either SGTP1 or GAPDH, and presumably a resultant aberrant glucose metabolism, did not exhibit any observable developmental defects in the time frames examined.

When we examined the duration of dsRNA treatments on SGTP1 transcript levels, we found that transcripts were still significantly reduced up to 28 days post-treatment. RNAi is similarly long-lived in other species. In the planarian Girardia tigrina the effect on targeted transcript was apparent up to 3 weeks post-injection as determined by in situ hybridization [6]. In C. elegans, where the mechanism and longevity of RNAi have been studied most extensively, phenotypes are often observed in progeny derived from dsRNA-injected adults, and in certain cases are observed in second and third generations [43]. For larval S. mansoni, the stability of the RNAi effect may make this method useful for studies on larval development, since daughter sporocysts (the second intramolluscan stage) production begins by day 20 [23,24]. Therefore, the effect of knocking down a specific gene during this developmental process could be directly assessed, a technology heretofore unavailable in any study of S. mansoni development.

We also found that dsRNA treatment was most effective when added within 1 h after the initiation of miracidial cultures. As the time between culture initiation and addition of dsRNA increased, the effectiveness of the treatment significantly declined, and at 24 h dsRNA addition was completely ineffective at reducing targeted transcript levels. One possible explanation for this disparity is that the shedding of the ciliary plates and formation of the new syncytial tegument, or outer surface of the organism, allows for a small “window” during which dsRNA is able to gain access to the inside of the developing larva. Once the tegument
has been formed, as it is in over 70% of larvae cultured for 20h, the dsRNA cannot enter in amounts sufficient to cause reductions in transcript levels. We tested this hypothesis by treating larvae with fluorescently labeled dsRNA as freshly hatched miracidia and after transformation into sporocysts (20h in culture), and did not observe any dramatic qualitative differences in fluorescence intensity or localization. While it was clear that miracidia display very little fluorescence barring punctate surface staining, no obvious differences in the distribution or amount of fluorescent label were observed between miracidia transformed to sporocysts in its presence and those treated with the label after 20h in culture (i.e. after sporocyst transformation). In both treatment groups, staining was detected throughout the bodies of certain larvae, within or associated with the tegumental surface in others, and in still others it was not detected at all, and this pattern of fluorescence that could be clearly correlated with differences in susceptibility to dsRNA treatment was not found. Moreover, we cannot be sure that the signal detected is entirely due to rhodamine associated with dsRNA, other than free rhodamine released by some enzymatic process in the culture medium or within the sporocyst itself, and therefore it is difficult to draw any conclusions from these data as to the tissues affected by applied dsRNA. They are merely suggestive that dsRNA entry is not likely to be the reason for the differences observed in the effect of dsRNA effect on transcript levels in these two life stages. Further work examining the fate of applied dsRNA, in both miracidia and sporocysts, will be necessary to address this question more completely.

Overall this study provides significant evidence that double-stranded RNA-mediated interference can be used to address questions related to gene function in larval stages of S. mansoni. As more DNA sequence information becomes available for schistosomes and other parasitic helminths, it will be crucial that suitable technologies exist for the reliable manipulation of genes of interest. This is particularly critical for parasitic flatworms, given that at present no effective genetic manipulation systems exist for use in this group of organisms comprised of a large number of important human pathogens.

Acknowledgements

The authors wish to thank Kate Zachman and Laura Johnston for expert technical assistance and Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD) for supplying infected mouse livers. Discussions on dsRNA delivery methodology with Dr. Judith E. Humphries (Iowa State University) were crucial to the success of this work. Special thanks to Dr. John Saven and members of the Saven laboratory (University of Wisconsin) for help in optimizing qPCR assays, as well as the reviewers who provided valuable comments on the manuscript. This research was supported by Individual NIH predoctoral NRSA No. MH12992 to J.P.B., NIH Grant Nos. AI38263 and AI15503 to T.P.Y. and NIAID schistosome supply Contract No. AI55270 to Dr. Lewis.

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