Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi*.

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Abstract

We describe the successful use of the reverse genetic technique RNA interference (RNAi) to investigate gene function in the human filarial nematode parasite *Brugia malayi*. We used fluorescently labelled double stranded RNA (dsRNA) to demonstrate that 300 bp molecules are able to enter adult females in culture while they remain excluded from microfilariae (mf). We have developed an optimised microvolume culture system to allow the exposure of parasites to high concentrations of dsRNA for extended periods. Culturing of adult female parasites in this system for 24 h does not significantly reduce parasite lifespan or mf release in culture. Three *B. malayi* genes, B-tubulin (*Bm-tub-1*), RNA polymerase II large subunit (*Bm-ama-1*) and *B. malayi* mf sheath protein 1/mf22 (*Bm-shp-1*) were targeted by soaking adult female *B. malayi* in dsRNA complementary to these transcripts in the optimised culture system. Targeting of the two housekeeping genes *Bm-tub-1* and *Bm-ama-1* led to a reduction in the levels of their transcripts, as assessed by reverse transcriptase coupled PCR (RT-PCR), and resulted in parasite death in culture. In contrast, targeting of the *Bm-shp-1* gene was not lethal to adult females in culture. A marked reduction in mf release was observed for *shp-1* RNAi parasites compared to controls and in addition 50% of mf released did not have fully elongated sheaths. This "short" phenotype correlated with the loss of the stockpiled *shp-1* transcript from developing mf in treated adult female gonads. From these data we conclude that RNAi may be a useful method for assessment of drug target potential of genes identified in filarial gene discovery projects.

Keywords: RNAi; *Brugia malayi*; *Caenorhabditis elegans*; Filarial nematode; Phenotype; Gene function

1. Introduction

For model, genetically tractable organisms such as the free-living nematode *Caenorhabditis elegans* [1], an array of genetic methods have been developed that permit hypothesis-driven investigation of function starting from sequence [2–4]. Many of these techniques, such as transgenesis [5], directed mutation [6] or gene replacement, are inappropriate for parasitic species with complex life-cycles, obligatory outcrossing and large brood sizes. Parasitic nematode genome projects, inspired by the success of the *C. elegans* program, have generated sequences for tens of thousands of genes [7–10]. Many of these genes have proved to be hard to annotate with functional descriptors, because they have limited similarity to genes from well-studied organisms. Even when genes can be annotated robustly through homology, it has been difficult to prove essential function in living parasites. We are interested in developing informatic and experimental methods for the identification of potential targets in nematodes that are only distantly related to the *C. elegans* model.

A new reverse genetic technology, based on the discovery that double stranded RNA (dsRNA) molecules derived from the same sequence as a targeted mRNA can result in specific and significant enzymatic degradation of the mRNA [11], has the promise of overcoming the current impasse in functional analysis of unknown genes [12]. This phenomenon, termed RNA interference (RNAi), was first experimentally demonstrated in *C. elegans* [13], but plays a role in many previously described gene silencing phenomena, and appears to be based on hijacking of a conserved cellular machinery that normally has a role in...
RNAi has been shown to be effective in mediating knockdown of mRNA levels of targeted genes in a wide range of organisms, from yeasts and plants to animals. Within the Metazoa, arthropod, nematode, cnidarian, platyhelminth and chordate species have been successfully tested. Many parasitic nematode species can be maintained outside their hosts in defined culture systems for significant periods of time. The strongylid gastrointestinal parasite Nippostrongylus brasiliensis was shown to be sensitive to RNAi by soaking in an in vitro system [20]. RNAi by soaking in the N. brasiliensis and C. elegans system requires large (300–500 bp) dsRNA triggers, and the major route of entry is via the mouth and gut. Unlike N. brasiliensis, many tissue-dwelling parasites, such as the L4 to adult stages of the filarial nematodes, are believed not to be active feeders, instead taking up much of the required nutrient through their cuticles [21–23]. It might be thought unlikely therefore that filarial nematodes such as Brugia malayi would be amenable to RNAi. We have tested B. malayi adult females for uptake of dsRNA and here show that it is indeed possible to achieve RNAi-mediated knockdown of known and novel targets in both adult females and their microfilarial progeny.

2. Methods

2.1. Standard culture of B. malayi for RNAi experiments

Adult female parasites, B. malayi TRS strain, were a kind gift from Prof. Rick Maizels and were taken from the life-cycle currently maintained by Ms. Yvonne Harcus at the Institute of Cell, Animal and Population Biology, Edinburgh. Parasites were removed from the peritoneal cavity of sacrificed jirds (Meriones unguiculatus) and washed once in culture medium RPMI 1640 (Invitrogen Life Technologies, UK), 1% penicillin/streptomycin (10,000 units/ml; Invitrogen Life Technologies, UK), 2% glucose; “standard” culture conditions hereafter) preheated to 37 °C. Parasites were then transferred to standard culture media in a total volume of 50–150 ml depending on the total number of parasites (about 5 ml per parasite). The medium was changed every 12 h for parasites being used for subsequent qualitative or quantitative analysis.

2.2. Culture of B. malayi in a microvolumetric dialysis system for exposure to dsRNA

The domed tops of 300 µl PCR tubes (Advanced Bio, UK) were removed with a sterile scalpel. A piece of dialysis tubing was inserted into the tube and the tube to form a barrier between the tube contents and the exterior. Tubes were filled with 330 µl of standard culture media, and groups of six, seven or eight healthy adult female parasites (assessed by high motility) were added with or without either fluorescein isothiocyanate (FITC)-labelled dsRNA or unlabelled dsRNA. Tubes containing parasites (Fig. 1B) were then placed in beakers containing 100 ml of standard culture medium preheated to 37 °C.

During the next 24 h period tubes were checked every 2 h to assess nematode health by motility. The contents of all tubes were mixed every 4 h. Parasites were transferred temporarily during these manipulations (which take approximately 5 min for each tube) to beakers containing standard culturing medium. The contents of the tube were removed and spun at 800 × g for 2 min in a bench top centrifuge to pellet released microfilariae (mf). The supernatant was then returned to the original tube, the nematodes replaced, and a fresh piece of dialysis tubing was placed over the top of the tube and the lid closed. Tubes were then returned to the original media containing beakers. In some experiments individual parasites were removed at required time points, rinsed briefly in PBS and frozen in Trizol reagent (Invitrogen Life Sciences, UK) for later preparation of nucleic acid. After 24 h the remaining parasites were placed in normal culture conditions for continued observation, except in the cases when they were already dead. When Brugia adults are placed in culture they are very active, and tend to congregate in “knots”. As the nematodes become unhealthy they first stop moving so vigorously and become extended and listless before dying, when they float in culture media. We thus scored adults as being “healthy” (in knots), “unhealthy” (extended but still motile) or “dead” (extended and unmoving even on mechanical stimulation).

2.3. Cloning of Bm-ama-1 and other target cDNA fragments and generation of templates for in vitro transcription

The B. malayi homologue of the C. elegans RNA polymerase II large subunit gene [24] was cloned by PCR from genomic DNA using primers directed against conserved peptide sequences identified in an alignment of nematode and other RNA polymerase II large subunit genes [25]. The degenerate primers used were GGAGARCCNGCAACD- CARATGAC (forward) and CCVACTYCTGHTGKRTTAKCC (reverse). The PCR conditions used were as follows: 95 °C for 1 min 1 × cycle; 95 °C for 25 s, 47 °C for 20 s, 72 °C for 1 min 35 × cycles; 72 °C for 10 min 1 × cycle. The DNA fragment obtained (995 bp) was sequenced,
Fig. 1. RNAi in *B. malayi*. Demonstration of dsRNA uptake by adult female nematodes and phenotypes of *shp-1*-treated microfilariae. (A) Adult female *B. malayi* were soaked for 18 h in FITC-labelled dsRNA at a concentration of approximately 80 μg/ml in the microvolume culture system described (Section 2). Fluorescence was visualised using a Zeiss Axiovert fluorescence microscope with emission at 520 nm. Staining is strongest at the mouth and vulval opening at the anterior of the nematode, throughout the nematode gut and in the gonadal system. (B) The microvolume dialysis chamber system for culture of *B. malayi* in high concentration of dsRNA. The Spectrapore membrane is sealed onto the top of the microtube (~300 μl volume) using a ring cut from the tube cap. The tube and nematode are immersed in the culture medium. (C–E) Approximately half the mf released by *shp-1* dsRNA-treated adult female parasites had a characteristic ‘short progeny’ Shp phenotype (D and E), rather the normal elongated sheath (C). The Shp phenotype could be graded in severity from mild (D) to severe (E). The proliferative phase of embryogenesis seems to be complete but embryos are unable to elongate and complete morphogenesis as the sheath has failed to elongate around them. This is probably due to loss of the SHP-1 protein which is normally incorporated into the mf sheath.

and analysis showed that it encompassed 850 bp of protein coding sequence split by 145 bp of intron. A fragment of the *B. malayi* beta tubulin gene was isolated using PCR primers designed from the published sequence [26]. The sheath protein 1 gene was isolated using PCR primers derived from the published genomic sequence [27, 28]. Approximately 300 bp coding fragments of the genes under investigation were cloned into a vector with T7 and T3 promoter sites (TA Topo cloning kit, Invitrogen Lifesciences, UK). Approximately 10 μg of DNA template was generated from these constructs by performing several 10 μl PCR reactions using T7 and T3 vector primers. The PCR conditions for amplifying each gene fragment for cloning were as follows: 95°C for 1 min 1× cycle; 95°C for 25 s, 55°C for 20 s, 72°C for 1 min 35× cycles; 72°C for 10 min 1× cycle. Forward and reverse primers respectively for each gene were as follows: *Bm-ama-1*, AGTCCA TGGCTGCGTA TC and CCACCA TCCGGAGTAA TCACAA; *Bm-tub-1*, ATATGCGTCCCAGGAGCA GT and CCGATACTCTCCACA TGAAAATT; *Bm-shp-1*, ACCAGGACAAGATA TGCAA CGG and GTTGG CATACA ATCTG CACCAC GC.

### 2.4. Generation of dsRNA and FITC-labelled dsRNA

FITC-labelled dsRNA was generated using FITC-conjugated uracil (Invitrogen Life Sciences, UK) and performing separate T7 and T3 polymerase (Invitrogen Life Sciences, UK) in vitro transcription reactions on 1 μg of template DNA according to the manufacturer’s protocol. Each reaction was treated with five units of RNase free DNase I (Invitrogen Life Sciences, UK) to remove template DNA. The reactions were then pooled, heated to 60°C for 5 min and allowed to cool at room temperature to allow annealing of complementary strands. A small aliquot was then run on a standard agarose gel to check for integrity of dsRNA. The FITC-dsRNA was then dialysed over RNase free water to remove free nucleotides and quantified using a spectrophotometer (Genequant, Abgene, UK).

Generation of large quantities of dsRNA using larger scale in vitro transcription reactions were performed using the Ambion MegaScript kit (Ambion, USA) according to the manufacturer’s protocol. Concentrations of PCR generated template producing the highest yield of RNA were...
2.5. Quantification of microfilarial release in culture

Quantification of mf release in culture was performed with either individual parasites or parasites pooled into groups. Individual nematodes were cultured for 1–3 h in 10 ml of standard culture medium in 15 ml Falcon tubes at 37 °C. The 10 ml of culture medium was then centrifuged in 15 ml Falcon tubes at 1100 × g for 10 min. The parasite had been removed to gently pellet all the released mf. The top 9.8 ml of medium was removed and the mf re-suspended in the remaining 200 µl aliquots of this remaining 200 µl under a binocular light microscope at 100× magnification. Counts were adjusted to represent the mf release per hour depending on the original collection time. Measurement of the release of mf from groups of parasites was performed in the same way except that parasites were cultured in 50 ml Falcon tubes in 40 ml of standard culture media.

2.6. Visualisation of microfilarial phenotypes released in culture

Microfilariae were collected for a set period of time, usually 3 h as described above. Collected mf were resuspended in standard culture media and 50 µl was placed on a glass microscope slide and spread gently using a 200 µl pipette tip. Slides were then left to air dry for 20 min and then fixed in 100% methanol for 5 min followed by rinsing in double-distilled and deionised H₂O. After slides had been allowed to dry for a further 10 min they were placed in Giemsa stain (Sigma, UK) diluted 1:10 in double-distilled and deionised H₂O for 20 min. Slides were washed again for 5 min in double-distilled and deionised H₂O. After being allowed to dry at 4 °C overnight slides were overlaid with large cover slips.

The fixed and stained slides were viewed under an inverted light microscope. The number of mf on the slide were counted and any unusual phenotypes scored and recorded. Pictures of microfilaria were taken using a colour digital camera and the Openlab 3.0 (Improvision, UK).

2.7. Single nematode RT-PCR

Single adult female nematodes were removed from culture and placed in Trizol reagent (Invitrogen Life Sciences, UK) and total RNA prepared according to the manufacturer’s protocol. The extracted total RNA was DNase I treated and used in a single oligo-dT primed reverse transcription reaction using the Stratagene High Fidelity RT-PCR Kit (Stratagene, UK). Oligo-dT primed reverse transcription excludes priming from the non-polyadenylated dsRNA used. The resulting 15 µl cDNA reaction was dialysed over double-distilled and deionised H₂O and re-concentrated to 12 µl using a DNA vacuum centrifuge. The expression levels of genes of interest were then assessed by 20 µl PCR reactions using 1, 2 and 3 µl of cDNA template. For ama-1, the PCR assay was directed against segments of the target mRNA external to the segment targeted by the dsRNA, and spanning introns in the gene. For shp-1 a second primer set was used that gave more robust single worm RT-PCRs. The tub-1 RT-PCR assay used previously published primers.

PCR reactions were optimised for each gene on untreated parasites, such that 3 µl of PCR product run on a 1.2% agarose gel gave strong bands when 3 µl of cDNA was used as template and progressively weaker bands when 2 or 1 µl was used. PCR conditions for each gene were as follows: Bm-ama-1, 94 °C for 5 min 1 ×; 94 °C for 15 s, 59 °C for 15 s, 72 °C for 30 s 31 ×; 72 °C for 3 min 1 ×; Bm-tub-1, 94 °C for 5 min 1 ×; 94 °C for 15 s, 58 °C for 15 s, 72 °C for 30 s 30 ×; 72 °C for 3 min 1 ×; Bm-shp-1, 94 °C for 5 min 1 ×; 94 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s 29 ×; 72 °C for 3 min 1 ×. Forward and reverse primers respectively for each gene were as follows: ama-1, TTACGCTGGGCCTTTGC and CATCAGATGATGCACAAATCCGGG; tub-1, ATATGTTCCACCCGACACGT and CGGATACTCCCTCAGAATT (the same primers used for the targeting construct); shp-1, GCATTGGCAT-TATGGTCGCAAG and GCACACTTTTITACACAGC (see Fig. 4E for a map of the primer positions).

2.8. Fluorescent microscopy of whole mount parasites

Single adult female parasites and both sheathed and exsheathed mf both in FITC-dsRNA for 18 h in the dialysis system described above were removed from culture medium and briefly rinsed in PBS. Parasites were then fixed for 5 min in 4.2% formalin and then placed on glass sides pre-treated with poly-lysine and allowed to air dry for 10 min. Slides were then overlaid with large coverslips and sealed with nail varnish (Chanel, France).
Parasites were viewed under mercury vapour lamp illumination through FITC (520 nm), DAPI (456 nm) and Texas red (620 nm) filters. Images were captured using a Hamatsu ORCA digital camera and Openlab 3.0. Autofluorescence of parasites (yellow-green at FITC wavelength) was apparently identical through all three wavelengths. This background fluorescence from the FITC image was eliminated by reducing the digital gain and exposure time of the camera such that autofluorescence at DAPI and Texas red wavelengths was eliminated.

2.9. Exsheathment of microfilariae in culture

Microfilariae were pooled and resuspended in 2 ml Hank’s buffered salt solution (HBSS; Invitrogen Life Technologies, UK) and allowed to stand for 5 min. To remove the proteinaceous sheath 2 ml of 2 mg/ml of Pronase solution (Sigma Protease type XIV from S. griseus suspended in HBSS) were added to a final concentration of 1 mg/ml [29]. Microfilariae were left to exsheath at room temperature in a 15 ml centrifuge tube for 10 min with gentle rolling. After 10 min, 400 µl of 10% fetal calf serum (FCS; Invitrogen Life Technologies, UK) was added. The mf were then centrifuged at 1100 x g for 5 min and washed twice in 9 ml HBSS and 1 ml of 10% FCS, centrifuging for 5 min after each wash. Microfilariae were washed for a final time in 10 ml HBSS. A sample of mf was transferred to glass slides and exsheathment checked by light microscopy. Around 90–95% of mf were successfully exsheathed by this treatment.

3. Results and discussion

3.1. Demonstration of uptake of FITC-labelled dsRNA by adult female B. malayi in culture

In order to develop a method to perform RNAi by soaking in B. malayi it was first necessary to demonstrate that dsRNA was taken up by parasites in culture. Molecules of slightly greater than 300 bp (including vector sequence) of dsRNA have been used in the experiments described here. Spermidine has been shown to enhance RNAi by soaking in C. elegans, although the mechanism for this is not known [30] and thus was included in the culture medium. FITC-labelled dsRNA, produced by in vitro transcription of a tub-1 cDNA [31], was taken up by adult parasites in culture (Fig. 1A).

Levels of fluorescence did not increase for periods of soaking beyond 18 h. Similar experiments were also performed on mf, both those released in culture and those collected directly from jird hosts, but they failed to show any internal fluorescence when soaked in FITC-dsRNA. To test whether this was due to the presence of the protective sheath that surrounds B. malayi mf the experiment was repeated with mf that had been exsheathed. However, exsheathed mf also failed to take up dsRNA in culture.

These data indicate that B. malayi adult females will take up dsRNA in culture. Whether this is a passive or an active process is not known. Staining appeared to be strongest at the mouth and vulval opening in the anterior of the nematode and along the gut. This is not surprising as these openings are likely to present the least resistance to the entry of macromolecules and perhaps the only route of entry. FITC fluorescence was also seen throughout the nematode, including in the gonad (not visible in Fig. 1A). It remains a possibility that the fluorescently labelled dsRNA was degraded into smaller fragments or even single nucleotides before being taken up by adult parasites.

3.2. Microfilarial release by adult female B. malayi in culture

Release of mf in culture by adult female B. malayi was investigated for two reasons. We wished to assess whether mf release rates could be used as a measure of deleterious affects, along with lifespan, of small volume culture methods. We also wished to use mf release as a robust phenotype to examine the effects of RNAi directed against genes required for mf development and release. All healthy individual adult females from three different jird hosts were cultured and their mf release counted for 3 h post-sacrifice. All three jirds used had been infected with infective L3 stage parasites between 8 and 14 weeks previously and had in excess of 80 healthy adult individuals (assessed by high motility in culture and including both sexes). Microfilarial release from these nematodes was normally distributed within populations taken from the same host (data not shown), but was different between populations from different hosts.

Thus, mf release in culture is robust enough to use as a measure of the deleterious affects of both small volume culture methods and RNAi as long as any comparative analysis between control and experimental conditions is carried out on parasites from the same jird host. Practically this limits its use as a phenotype to experiments that require up to 40 individual adult female parasites.

In order to expose adult female parasites to high concentrations of dsRNA in culture we developed a low-volume culture system. We developed a system partitioned across a dialysis membrane allowing culture in large effective volumes of low molecular size solutes such as glucose while maintaining high concentrations of dsRNA local to the nematode. Two measures of parasite health were used. Survival time of adults in culture was assessed by direct observation: nematodes were considered to be unhealthy if they displayed reduced motility and began to uncoil and straighten out. This phenotype normally indicates that death will occur within 72 h (unpublished observations). Nematodes were...
considered dead when they no longer moved and floated in culture media.

Culture of parasites within the optimised microvolume dialysis system for 24 h resulted in a slight, but not significant, reduction in lifespan when seven or fewer were used (Fig. 2A). This reduction was however consistently observed and could be due to the limits of diffusion set by the relatively small area of the dialysis membrane contact between the media in which the nematodes are kept and the surrounding media. This problem could be circumvented by designing a holding vessel allowing greater contact with the surrounding media. When eight or more adult females were used a significant reduction in lifespan was observed (Fig. 2A), suggesting that this number is too many to maintain their health in this system.

Release of mf was also measured for parasites cultured in groups of seven for the days proceeding the 24 h period in the microvolume dialysis system (Fig. 2B). The mf release immediately post-sacrifice of all the individual parasites in the control groups and those cultured in groups of seven within the dialysis system was measured. Adult parasites with mf releases close to the mean of the whole population from each individual jird were pooled into groups so that each group had an equivalent average mf release frequency. After culturing for a further 24 h (in either normal or dialysis conditions) mf release for each group was measured again to give the mf release average after 2 days post-sacrifice. This was repeated every 24 h for each group until the first unhealthy nematode was observed. A slight decrease in mf release was observed for those parasites maintained in the dialysis culture system for the first 24 h post-sacrifice (Fig. 2B).

Taken together these data indicate that the dialysis system used here permits small volume culture of B. malayi adult females. In addition it demonstrates that both lifespan and mf release in culture are robust phenotypes for preliminary assessment of RNAi experiments.

3.4. Choice of target genes for RNAi in B. malayi

Three genes were chosen as preliminary targets for RNAi in B. malayi. Two of these, RNA polymerase II large subunit (Bm-ama-1, the homologue of the C. elegans ama-1 gene, which can be mutated to give alpha amanitin resistance [24]; cloned during this study; see Section 2) and \( \beta \)-tubulin (Bm-tub-1; [26]), were selected because they are expected to be essential for survival. \( \beta \)-Tubulin is a known drug target (for benzimidazoles and derivatives) [32], while RNA polymerase II is essential in many organisms. Reduction of transcript levels of these genes was predicted to adversely affect nematode health. The third gene selected was that coding for the microfilarial sheath-protein-1 (Bm-shp-1) [27,33–35]. This transcript is highly abundant and expressed only in adult females. Loss of mRNA, leading to loss of protein, was predicted to affect the structure of the mf sheath, a phenotype that could be measured in the microfilaria released in culture by adult female parasites. Coding fragments of each gene were cloned and used as templates for PCR reactions with T7 and T3 primers to produce template DNA for in vitro transcription reactions. RT-PCR reactions on panels of individual nematodes were performed (data not shown) for each gene to show that the optimised PCR conditions gave consistent results.

3.5. RNAi of B. malayi \( \beta \)-tubulin and RNA polymerase II large subunit genes results in reduction of transcript levels and death of adult female nematodes in culture

Adult females from the middle of the mf release distribution were pooled into five groups of seven nematodes each so that each group had approximately the same mean mf release count. The first group of nematodes was placed in standard culture conditions for the duration of the experiment, the second group was placed within the microculture system for the first 24 h and the remaining three groups were soaked in dsRNA corresponding to one of the genes described above in the microculture system. Concentrations of dsRNA were 3.7 mg/ml (ama-1), 3.6 mg/ml (tub-1) and 3.5 mg/ml (shp-1). Nematodes were removed after 10, 14, 17, 20 and 24 h in the microculture system to assess transcript levels by single nematode RT-PCR (Fig. 3).

Control nematodes showed normal transcript levels at all time-points and were observed to be healthy throughout the 24 h period with only a small reduction in motility (Fig. 3). This reduction in motility was reversed when parasites were placed back into standard culture conditions. However, transcript levels of both ama-1 and tub-1 were reduced during the 24 h period by soaking in complementary dsRNA (Fig. 3).

In nematodes treated with dsRNA to ama-1, ama-1 transcript levels started to drop between 14 and 17 h (Fig. 3), at which time the remaining nematodes appeared unhealthy and showed reduced motility compared to control nematodes. By comparison with the data obtained from PCR optimisation experiments we estimate that this drop represents loss of two thirds of normal transcript levels. The transcript level had further reduced at 20 h and nematodes were observed to have further diminished motility. At this time a significant drop in tub-1 transcript (assessed as an internal control) was also observed but levels of shp-1 dropped only very slightly. As would be expected, loss of RNA polymerase II protein causes a general reduction in transcript levels. Detection of shp-1 transcript by RT-PCR were possibly less affected as high levels of this transcript are synthesised and stockpiled in the embryos in utero [28]. After 24 h the remaining nematodes were scored as dead as they showed no motility and failed to recover on transfer to normal culture conditions. RT-PCR at this time point did not detect any transcript for any of the genes.

In the nematodes treated with dsRNA from tub-1, the level of tub-1 transcript started to drop between 10 and 14 h, and was just detectable after 20 h (Fig. 3). However, transcript levels of the non-target genes also dropped to just detectable levels after 20 h. ama-1 levels dropped after tub-1 levels...
Fig. 2. *B. malayi* survival and fecundity in microvolume dialysis culture. (A) The effect of microvolume dialysis culture on survival of adult females. Three groups of adult female parasites taken from three different jird hosts were split into groups and cultured in two different conditions. Parasites were grouped on the basis of their mf release such that each group had the same mean mf release per adult female per hour. Nematodes were either cultured in standard conditions (S) or in the dialysis system (D) as described in groups of 8, 7 or 6. They were maintained in these culture conditions for the first 24 h post-sacrifice and then moved to standard culture conditions. Nematode health was then observed by assessing motility, which is characteristically very vigorous. The day post-sacrifice on which the first adult in a group was scored as unhealthy was recorded as well as the day on which the first adult in a group was scored as dead (no motility and floating). Culturing less than eight nematodes in the dialysis system does not significantly affect survival. (B) Comparison of microfilarial release after standard or microvolume dialysis culture. Release of mf was assessed for groups of parasites cultured in either standard conditions (standard) or the microvolume dialysis system (dialysis) for the first 24 h post-sacrifice from three different jird hosts. Release of mf from nematodes maintained in the dialysis system was consistently lower than that of nematodes maintained in the standard system. However, despite this drop in mf release the difference is not large enough to preclude mf release as a measure of the effect of RNAi and suggests the effect of dialysis culture on health as measured by mf release is only slight.
Fig. 3. Single nematode RT-PCR to assess transcript levels in control and RNAi-treated *B. malayi*. Groups of seven adult female *B. malayi* were exposed to high concentrations of dsRNA for 24 h post-sacrifice. After 10, 14, 17, 20 and 24 h individual nematodes were removed from each group and stored for later RNA and first strand cDNA preparation. First strand cDNA was prepared for each worm and the level of *ama-1*, *shp-1* and *tub-1* transcripts assessed for each nematode as described (Section 2). Transcript levels of all genes were normal for control nematodes (panel A) not exposed to dsRNA. Target-specific reduction in transcript levels was observed for nematodes taken from the groups exposed to dsRNA (described in the text). The concentrations of dsRNA that each group was exposed to were as follows: 3.6 mg/ml of *ama-1* dsRNA (panel B), 3.7 mg/ml of *tub-1* dsRNA (panel C), 3.5 mg/ml of *shp-1* dsRNA (panel D). After 24 h the remaining nematodes treated with *tub-1* and *ama-1* dsRNA were dead and no RT-PCR signal was detected for any genes. The position of the dsRNA (double dashed lines) and RT-PCR fragments (double solid lines) on the targeted mRNAs is shown in panel E. The positions of the extent of each fragment are given in base pairs. The triangles indicate that the fragments span intron(s) in the corresponding gene.

3.6. RNAi of the *B. malayi* shp-1 gene results in specific transcript reduction and the production of “short” microfilaria in culture

RNAi of *shp-1* did not result in nematode death, although the nematodes remaining at the end of the 24 h in the dialysis bag would be used in screens to identify putative lethal drug target candidate genes.
system showed markedly reduced motility on their return to standard culture conditions compared to control nematodes. Reduction in shp-1 transcript levels began between 10 and 14 h and continued to decrease until they were no longer detectable at 20 and 24 h (Fig. 3). Transcript was detectable when more sensitive PCRs (35 cycles of amplification) were performed. RNAi experiments in C. elegans suggest that for highly expressed genes low levels of transcript can still be detected by RT-PCR even after effective RNAi [36]. Importantly, no transcript reduction was observed for either of the non-target genes. We conclude that, unlike ama-1 and tub-1, shp-1 is not essential for parasite survival in culture in the short term, perhaps because the protein translated from this maternal/embryonic transcript appears only in the mf sheath [27,33–35].

In order to further investigate the function of shp-1 and the dynamics of RNAi knockdown persistence in this system the remaining nematodes at the end of the 24 h period were moved to standard culture conditions. Microfilarial release counts were performed on both control and shp-1 RNAi nematodes immediately after removal from the dialysis system, and again after a further 24 and 48 h. The mf release counts for the shp-1 RNAi nematodes were lower than those of control nematodes, which in turn were only slightly lower than the counts for nematodes that were not placed in the microculture system (Table 1). Released mf were fixed and Giemsa stained to observe the phenotypes of their sheaths. Half of the mf released by shp-1 RNAi nematodes in standard culture after 48 h were phenotypically distinct, with malformed sheaths (Table 1, Figs. 3 and 4). This was characterised by apparently late-stage, apparently mature mf coiled up within sheaths that had failed to elongate to greater or lesser extents. The phenotype suggests that loss of shp-1 transcript in the gonad of adult female parasites interrupts normal elongation of the embryo eggshell into the mf sheath. The filarial gonad can be likened to a production line, and thus even in RNAi-treated nematodes there will be mf that have completed much of embryogenesis. Thus, normal mf released early in culture from treated nematodes probably represented these more mature embryos; increasing numbers of affected embryos were released later. It is not clear whether the reduction in total mf release was due primarily to loss of shp-1 transcript or due to some secondary effect on viviparous mf release. Some aberrant mf release was also observed from control nematodes but these were examples where embryogenesis was not complete due to early errors in development (Table 1).

SHP-1 belongs to a group of proteins either known to or supposed by analogy to incorporate into the mf sheath structure [33]. The shp-1 gene itself is known to be an entirely maternally/embryonic derived transcript as the transcript is absent from mature mf. Work on shp-1 from Litomosoides sigmodontis (a closely related filarial nematode, previously known as Litomosoides carinii) indicates that this transcript is present in large amounts within embryos during development while no protein is detected [35]. During development and elongation of the mf sheath, translation of stockpiled transcript occurs and mature SHP-1 protein is moved to the eggshell/mf sheath. In both species its structure suggests that it may contribute to the architecture of the mf sheath.

Motility observations on elongated mf collected in culture from untreated and shp-1 RNAi adults had apparently equal motility and flexibility by eye under light microscopy. Abnormal mf that were not elongated had either no or very low motility, except for rare individuals that had undergone
4. Conclusions and prospects

From the data presented here we can conclude that RNAi using externally applied dsRNA (“RNAi by soaking” [17]) works in the filarial parasite B. malayi and therefore that the molecular machinery of the RNAi process identified in model organisms is conserved. We suggest that the effects were specific to the genes being targeted rather than being due to a general detrimental effect of the culture system and dsRNA exposure. dsRNA is not known to be non-specifically lethal or to induce non-specific phenotypes in other systems. We did observe some reduced motility in dsRNA-treated nematodes compared to controls incubated in medium alone, but this was not significant. In nematodes, RNAi has previously been demonstrated in the free-living rhabditids C. elegans and Caenorhabditis briggsae [12,37], the animal-parasitic strongyloid N. brasiliensis [20], and the tylenchid plant-parasitic Heterodera glycines and Globodera pallida [38], but some species, and some isolates of C. elegans have proved refractory to RNAi by soaking. The gut parasite N. brasiliensis can be maintained relatively well in culture, and RNAi knockdown of acetylcholinesterase genes was shown to effect secreted protein levels and nematode maintenance in the host [20]. In the microvolume dialysis system for RNAi in culture described here we were able to target B. malayi genes with both adult function and those required for proper formation and release of mf in culture. These two classes of genes are of primary interest in the search for macrofilaricidal and microfilaricidal drug targets.

Further characterisation of the dynamics of RNAi in B. malayi would improve its utility for studying parasite gene function. Are all adult and microfilarial transcripts accessible, or will there be exclusion from some tissues, such as the nervous system as observed in C. elegans [13]? From the shp-1 transcript data we conclude that RNAi efficiently targets the gonad and that embryonic and microfilarial processes should be accessible. It would be advantageous to be able to test nematodes compromised by RNAi knockdown in well-established in vitro and in vivo biochemical and immunological assays for looking at gene function. For this, the RNAi knockdown effect would have to be relatively persistent in the absence of dsRNA trigger. Many immunological models of filariasis require that adult nematodes are reimplanted into hosts so that the effects on the host immune response can be investigated. Ideally RNAi could be used to target the expression of possible immune regulatory candidates produced by the parasite [39–42]. However, to look at the immunobiology of infections any RNAi effect would have to be maintained for several weeks. shp-1 RNAi nematodes still had extremely reduced transcript levels at 48 h post-treatment. In the N. brasiliensis system, optimisation of RNAi conditions permitted functional analysis of reimplanted adults. If long-term knockdown with RNAi proves effective then both the long-term and short-term host parasite interactions involved in parasite establishment and survival might be illuminated.

As has been amply demonstrated in C. elegans, RNAi by soaking can enable functional analysis of genome-scale datasets [36,43–46]. Most parasitic nematodes are intractable to experimental genetics, and DNA transformation has been achieved with only transitory effect in a very few species [47–49]. With the rapid progress of the B. malayi and other nematode genome projects, dsRNA-mediated RNA interference may aid identification of novel drug targets from the large numbers of otherwise unannotated genes sequenced from these important pathogens.

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