Brugia malayi: transient transfection by microinjection and particle bombardment

Tarig B. Higazi, Anthony Merriweather, Limin Shu, Richard Davis, and Thomas R. Unnasch*

Abstract

To develop a method for the introduction of DNA into filarial parasites, several methods that have proven successful in other organisms were evaluated for their ability to transform Brugia malayi. Luciferase activity was detectable in embryos bombarded with gold particles coated with a construct consisting of a luciferase reporter gene under the control of the 5S rRNA intergenic spacer (SL promoter). Similar results were seen in adult parasites and infective larvae bombarded with this construct, or in adult female parasites microinjected with the plasmid. In similar experiments employing the SL promoter driving a green fluorescent protein (GFP) reporter, expression of the reporter was detectable in the intrauterine embryos of the microinjected adult parasites, and in the sub-cuticular tissues of biolistically transfected adult female parasites. A similar pattern of GFP expression to that seen in the SL promoter construct transfected parasites was noted in parasites transfected with constructs consisting of the upstream domain derived from an aspartyl aminoacyl tRNA synthetase gene of B. malayi. The ability to transfec B. malayi embryos may provide a foundation for studies of the regulation of gene expression and function in these organisms.

Index Descriptors and Abbreviations: filarial nematodes; spliced leader; particle bombardment; microinjection; bp, base pairs; EST, expressed sequence tag; GFP, green fluorescent protein; L3, Infective larvae; nt, nucleotides; PCR, polymerase chain reaction; rRNA, ribosomal RNA; SL, spliced leader © 2002 Elsevier Science (USA). All rights reserved.

1. Introduction

The advent of the polymerase chain reaction and the development of more efficient methods of molecular cloning and library construction have resulted in a rapid accumulation of knowledge concerning the genes expressed by human parasitic filaria. The Filarial Genome Project has been instrumental in this effort, having generated sequence data on over 30,000 expressed sequence tags (ESTs) from all three of the major human filarial parasites (The Filarial Genome Project, 1999). cDNA cloning and expression methods have also made it possible to characterize specific parasite proteins that are involved in the development of protective immunity against filarial infections (Abraham et al., 2001; Folkard et al., 1997; Jenkins et al., 1998; Joseph et al., 1997), and in the development of pathology (Pearlman et al., 1997, 1994; Tawe et al., 2000). However, nothing is known about how these parasites regulate their gene expression.

Perhaps the most striking deficiency in the study of the filarial parasites is the inability to genetically manipulate these organisms. This problem is likely to become even more acute as time goes on. For example, the Filarial Genome Project is moving from studies based on EST analysis and gene discovery to studies of the structure of the filarial genome itself. An important outcome of these studies will be the potential of identifying cis and trans acting elements important for the regulation of gene expression.
However, the extent of these studies will necessarily be limited by the lack of genetic methods to manipulate these organisms.

It has not been possible to carry out conventional genetic studies in the human filaria, as it has been impossible to isolate mutants with easily scored phenotypes or to carry out defined genetic crosses. In the absence of classical genetics, reverse genetics can be used to address many questions regarding the function and regulation of various genes. Reverse genetics involves the introduction of a gene sequence into an organism, either a wild type allele or a mutant constructed through in vitro techniques. In the free living nematode Caenorhabditis elegans reverse genetic approaches have proven to be exceptionally useful in investigating developmental pathways and in studying the phenotypic effect of gene disruption. However, similar techniques have not been developed for human filarial parasites.

It has long been known that C. elegans can be transfected by microinjection of circular DNA directly into the gonad of the hermaphrodite (Stinchcomb et al., 1985). In general, the injected sequences are not incorporated into the C. elegans genome; rather they form large extrachromosomal arrays consisting of head to tail concatamers of the injected circular molecules (Stinchcomb et al., 1985). These arrays, although extrachromosomal, are capable of being inherited resulting in the development of stably transfected worm lines (Stinchcomb et al., 1985). Furthermore, although the majority of the progeny of the microinjected worms maintain the transgenic sequences as extrachromosomal arrays, occasionally the transgenic sequences are incorporated into the genomic DNA. Microinjection has also been used to transfet the entomopathogenic nematode Heterorhabditis bacteriophora (Hashmi et al. 1995a,b). In these studies, roughly 8% of the progeny of the transfected worms expressed the transgene, suggesting that microinjected sequences persisted for at least one generation.

A very versatile method for the introduction of exogenous DNA is particle bombardment or biolistics. Biolistics has been shown to be capable of transfecting intact C. elegans (Jackstadt et al., 1999; Wilm et al., 1999). As seen with animals transfected by microinjection, biolistically transfected animals are capable of transmitting the transgenes to their progeny. Recently, studies have demonstrated that isolated embryos of Ascaris suum can also be transiently transfected employing biolistics (Davis et al., 1999).

In the current study, biolistics and microinjection have been evaluated as potential methods for transfection of the human filarial parasite Brugia malayi. We demonstrate that either method may be used to transiently transflect this parasite, opening the way for studies of gene expression in this organism.

2. Materials and methods

2.1. Construction of expression reporter plasmids

Two sequences encoding putative promoter domains were chosen for inclusion in this study. The first was the intergenic spacer domain of the 5S rRNA gene cluster. This region of DNA has previously been shown to encode the promoter controlling transcription of the spliced leader pre-mRNA in A. suum (Hannon et al., 1990a), and was found to be an effective promoter in the A. suum transient transfection system (Davis et al., 1999). The second sequence chosen was the upstream domain derived from a 65 kDa protein that was originally described as a potentially protective antigen from B. malayi (Perrine et al., 1988). Subsequent analyses revealed that this protein probably represented an aspartyl tRNA synthetase homologue (Kron et al., 1992).

Primers were developed to amplify the two putative promoter domains from genomic DNA samples of B. malayi and O. volvulus. The primers used to amplify the 5S intergenic regions containing the putative spliced leader promoter were 5′CAAGCTTATCCCCAGGTGTTGTAGACAT3′ and 5′CCAAGCTTGAAACATTCAATTACCTCAAC3′. These primers were constructed with synthetic HindIII sites to facilitate subsequent sub-cloning. The sequences of primers used to amplify the putative B. malayi aspartyl tRNA synthetase gene promoter were 5′ACCGATCTTCTCAAGATATAGTAA3′ and 5′TACAAACAACCTTCTCTCAATTAAT3′. Amplification reactions contained 20 μM Tris–HCl (pH 8.0), 2 mM MgCl2, 10 mM KCl, 6 mM (NH4)2SO4, 0.1% Triton X-100, 1 μg/ml bovine serum albumin, 200 μM each dATP, dCTP, dGTP, and dTTP, 0.5 μM of each primer, 5 U of Pfu1 polymerase (Stratagene), and 10 ng of parasite genomic DNA. Amplification conditions consisted of 35 cycles of 1 min at 94 °C, 1 min at 48 °C, and 3 min at 72 °C. The resulting amplification products were purified, A tailed as previously described (Tang et al., 1996), and cloned into a Tailed PCR cloning vector (Original TA cloning vector, Invitrogen). The DNA sequence of the resulting cloned PCR amplification products was confirmed and the cloned PCR product was excised from the TA cloning vector. In the case of the 5S constructs, the insert was excised with HindIII. The aspartyl tRNA synthetase promoter was excised from the TA cloning vector by digestion at the EcoR1 sites directly flanking the cloning site. The purified inserts were then cloned into the reporter plasmids. For luciferase reporter constructs, the plasmid backbone used was pGL3 basic (Promega). The 5S fragments were cloned directly into the HindIII site of the polylinker in pGL3, while the aspartyl tRNA synthetase fragment was cloned into a vector in which the XhoI site of the polylinker had been converted to an EcoRI site. For GFP constructs, the plasmid backbone
was pPD95.75. This plasmid consists of a GFP reporter gene with five synthetic introns cloned downstream of a multiple cloning site for promoter insertion. pPD95.75 was a kind gift of Dr. Andrew Fire. The DNA sequence of pPD95.75 is available from ftp://ciw1.ciwemb.edu/pub/FireLabVectors/Vector97/Vec97Txt. The B. malayi 5S fragment was directly cloned into the HindIII site of pPD95.75. For the aspartyl tRNA synthetase fragment, the EcoRI ends were converted to blunt ends by end filling and the resulting blunt end fragment was cloned into HindIII digested and end filled pPD95.75.

3. Biolistic transfection

Developing embryos were isolated from gravid adult females by dissection. Plasmid DNAs containing the various promoter–reporter gene constructs were purified by cesium chloride gradient centrifugation and used to coat 0.6 μm gold particles (BioRad #1652262) according to the manufacturer’s instructions. In brief, gold microcarriers were soaked in 70% Ethanol, washed three times in sterile water, and stored at 4 °C in 50% glycerol at a final concentration of 30 mg/ml. The carrier solution was subjected to vigorous mixing at room temperature for 15 min prior to use. To coat the microcarriers, the following were added to a 1.5 ml microcentrifuge tube while the tube was mixed continuously on a vortex mixer: 50 μl gold solution, 5 μl plasmid DNA (at 1 mg/ml) calcium chloride to a final concentration of 1 M, and free base spermidine (Sigma #S-3256) to a final concentration of 0.015 M. The coated beads were washed once following the addition of 1 M, and free base spermidine (Sigma #S-3256) to a final concentration of 0.015 M. The solution was agitated for 3 min following the addition of spermidine. The coated beads were washed once with 140 μl 70% ethanol, once with 140 μl absolute ethanol, and resuspended in 18 μl absolute ethanol. A total of 6 μl (0.5 mg) of coated gold particles was used for each transfection. The coated gold particles were used to bombard adult parasites and isolated embryos in a Biolistic unit (BioRad, Hercules, CA). Isolated embryos and L3 Larvae were bombarded at the pressure of 1100 psi under a vacuum of 12.5 in. of mercury, while adults were bombarded under similar conditions at a pressure of 2200 psi. Individual adult female parasites, approximately 150 L3s or roughly 1 x 10^5 isolated embryos, were used in each bombardment. Following bombardment, the parasites were allowed to rest in a humid chamber for 5 min, and flooded with CF-RPMI tissue culture medium (RPMI tissue culture medium containing 25 mM HEPES, 20% fetal calf serum, 20 mM glucose, 24 mM sodium bicarbonate, 2.5 μg/ml amphotericin B, 10 U/ml penicillin, 10 U/ml streptomycin, and 40 μg/ml gentamicin). The parasites were maintained in CF-RPMI at 37 °C under an atmosphere of 5% CO₂ for 48 h, unless otherwise noted.

4. Microinjection of adult female parasites

Microinjection needles were prepared from commercially available capillaries (Quick seal Glass, World Precision Instruments, Item# 1B100F-4). Needles were pulled using a Kopf needle/pipette puller model 730 (David Kopf Instruments, Tujunga, CA) at the following settings: Heat 1 = 12, heat 2 = 0, solenoid = 1.4. Prepared microinjection needles were loaded with DNA constructs at a concentration of 100–200 μg/ml in microinjection buffer (2% w/v polyethylene glycol, 20 mM potassium phosphate (pH 7.5), 3 mM potassium citrate). Adult female B. malayi were immobilized by chilling on ice for 5 min, transferred to an agarose pad, and covered with heavy paraffin oil (BDH Chemicals, England) to prevent desiccation. Agarose pads were prepared by spreading a 2% solution of melted agarose containing crushed glass between two 24 x 50 mm coverslips and allowing the agarose to solidify. The coverslips were then separated, dried overnight at room temperature, and stored. Each worm was injected at several spots by pushing the needle through the body into the uterus. The DNA solution was then ejected from the needle by release of helium gas at 30 psi. Injected worms were recovered by adding a few drops of CF-RPMI medium and transferring them into fresh medium. Worms were then incubated at 5% CO₂, 37 °C, and 100% humidity for 48 h.

5. Analysis of transfected parasites

Parasites transfected with constructs containing a luciferase reporter gene were collected into a chilled microglass homogenizer, frozen at −80 °C, and homogenized while frozen to a powder. A total of 100 μl 1X cell culture lysis reagent (Promega, Madison, WI) was added to each homogenizer and the homogenization process continued. Homogenates (40 μl) were then assayed for luciferase activity using the luciferase assay reagent provided by Promega, following the manufacturer’s instructions. Light units were measured as counts per second in a Packard TopCount NXT microplate luminometer. Protein content in each extract was determined using the Bradford method with reagents provided by BioRad. Results were expressed as net light units (gross counts per second minus the average of two negative control assays) per milligram of protein.

Worms transfected with GFP reporter gene constructs were fixed in 100% methanol at −20 °C for 1 h and examined using a Leica DMRBIE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT Laser Confocal optics. To image GFP, the 488 nm line of the argon laser was used for excitation, and the prism spectrophotometer was set for a broad emission bandpass of 490–600 nm.
6. Results

Previous experiments had demonstrated that the spliced leader (SL) promoter encoded in the intergenic spacer of the 5S rRNA gene cluster was found to drive the expression of a luciferase reporter gene in *A. suum* embryos (Davis et al., 1999). As the first step in determining if biolistics could be used to transfect *B. malayi*, the 5S rRNA intergenic spacer of *B. malayi* was isolated by PCR and cloned upstream of the luciferase reporter gene in pGL3 basic. The resulting plasmid (pBm5S-luc) was then precipitated onto 0.6 μm gold particles and used to bombard the isolated embryos. Luciferase activity was detectable in the bombarded parasites, with gross lightunits in the bombarded samples ranging from 2 to 10-fold over background levels in untreated worms, or worms bombarded with beads alone (data not shown). The background levels in unbombarded parasites were less than or equal to the background level of assays containing no worm extracts (data not shown). The background levels in unbombarded parasites were less than or equal to the background level of assays containing no worm extracts (data not shown). Luciferase activity was evident within 24 h of bombardment, and remained at relatively high levels for roughly 72 h postbombardment (Fig. 1). The activity was found to decrease markedly after 72 h, although some activity was still evident 5 days after bombardment. Viability of the embryos cultured for five days remained above 70%, as measured by propidium iodide/ethidium bromide staining (Live/Dead assay, Molecular Probes), and motile pretzel microfilaria continued to be visible within the eggshells of the cultured embryos throughout the five days in culture.

The luciferase activity seen in the transfected embryos was dependent on the presence of the putative *B. malayi* promoter sequence. Embryos bombarded with beads carrying the pGL3 vector without the SL promoter sequence produced low levels of luciferase activity (Fig. 2). In contrast, the enzyme specific activity in parasites bombarded in parallel with pBm5S-luc was roughly 20 times greater than that seen with the vector alone (Fig. 2).

The sequences of 5S intergenic spacer domains located upstream from the spliced leader pre-RNA vary greatly among the filarial nematodes (Xie et al., 1994). It was therefore of interest to determine if the 5S spacer domain from another filarial nematode might function as a promoter in *B. malayi*. To accomplish this, the 5S intergenic spacer region was amplified from *Onchocerca volvulus* resulting in the production of a 146 bp fragment. The DNA sequence of intergenic regions of *B. malayi* and *O. volvulus* exhibited little similarity outside of the 5S rRNA gene and spliced leader sequences (Fig. 3). Despite this, when the *O. volvulus* 5S region was cloned into pGL3 (producing construct pOv5S-luc) and used to bombard isolated *B. malayi* embryos, the embryos produced luciferase activity levels that were equal to or greater than those seen in embryos bombarded in parallel with the homologous *B. malayi* construct (Fig. 4).

Constructs containing a downstream trans splice acceptor enhanced the luciferase activity in the *A. suum* transfection system (Davis et al., 1999). To determine if this was the case in *B. malayi*, a 105 bp PCR fragment containing the splice acceptor sequence from the vacuolar ATP synthase gene from *A. suum* (Hannon et al., 1990b) was placed downstream of the *B. malayi* spliced leader promoter. The construct containing the splice acceptor (pBm5S-sa-luc) produced a level of luciferase activity that was roughly 9-fold greater than that produced by a construct containing the same promoter, but lacking the splice acceptor (Fig. 5).

The successful transient transfection of isolated embryos suggested that biolistics might also be used to introduce exogenous DNA into intact parasites. To test
this hypothesis, intact adult female parasites and infective larvae were subjected to bombardment with pBm5S-sa-luc. Bombarded parasites maintained mobility until being harvested for luciferase activity determinations. Luciferase activity was detected in both L3 and adult female parasites, although at lower specific activities than seen in preparations of biolistically transfected embryos (Fig. 6).

Intrauterine microinjection has been the standard method for the production of stable transfectants in the free-living nematode *C. elegans* (Mello et al., 1991). To determine whether this technique could be successful in transfecting *B. malayi*, uteri of adult female parasites were injected with pBm5S-sa-luc. Because it was im-

---

**Fig. 3.** Sequence comparison of 5S rRNA intergenic spacers of *B. malayi* and *O. volvulus*: The overlined sequences represent the conserved sequences derived from the 5S rRNA (5′ end) and the domain located downstream of the spliced leader sequence (3′ end). Double overlining indicates the sequence of mature spliced leaders. Vertical lines indicate identical residues and dashes indicate gaps introduced to maximize the alignment. The sequences of 5S rRNA gene repeats are available under accession numbers D87037 (*B. malayi*) and U09024 (*O. volvulus*).

---

**Fig. 4.** Comparison of luciferase activity in embryos transiently transfected with pBm5S-luc and pOv5S-luc: Parasites were bombarded with pBm5S-luc or pOv5S-luc coated beads and maintained in culture for 48 h prior to being assayed for luciferase activity. Values shown represent means of duplicate determinations and error bars indicate the range of duplicate values.

---

**Fig. 5.** Luciferase activity in parasites transfected with 5S constructs with and without a 5′ splice acceptor: Parasites were bombarded with pBm5S-luc or pBm5S-sa-luc coated beads and maintained in culture for 48 h prior to being assayed for luciferase activity. Values shown represent means of duplicate determinations and error bars indicate the range of duplicate values.

---

**Fig. 6.** Luciferase activity in adult parasites, L3 or isolated embryos transfected with pBm5S-sa-luc: Parasites were bombarded with pBm5S-sa-luc coated beads as described in Section 2, maintained in culture for 48 h, and assayed for luciferase activity. Values shown represent means of duplicate determinations and error bars indicate the range of duplicate values.
possible to identify the gonadal syncytium, parasites were injected in the anterior, medial, and posterior areas of the uterus. Parasites injected in all three regions of the uterus produced detectable levels of luciferase activity (Fig. 7). Parasites tolerated the procedure well, remaining motile until being harvested, although prolapse of the uterus through the injection site was noted in some worms. The specific activity of the microinjected parasites was higher than that seen in the biolistically transfected adult females, but less than that seen in isolated embryos biolistically transfected with pBm-sa-luc (Fig. 7).

Because it was necessary to homogenize parasites transfected with the luciferase reporter constructs, it was impossible to determine what stages or tissues of the transfected parasites were expressing the transgene. To answer this question, the 5S promoter was cloned into a GFP reporter vector pPD95.75. The resulting construct (designated pBm5S-GFP) was then used to transfect intact adult females by microinjection and biolistic bombardment. Parasites injected with pBm5S-GFP expressed GFP in the individual intrauterine embryos in the vicinity of the injection site (Fig. 8, panel A). In contrast, parasites bombarded with pBm5S-GFP

![Image](Fig. 7. Luciferase activity in adult parasites microinjected with pBm5S-sa-luc: Parasites were microinjected with pBm-5S-sa-luc as described in Section 2, maintained in culture for 48 h and assayed for luciferase activity.)

![Image](Fig. 8. GFP expression in biolistically transfected and microinjected adult parasites: Parasites were transfected with various constructs, maintained in culture for 48 h, and GFP expression was visualized as described in Section 2. Panel A: Parasite microinjected with pBm5S-GFP. The arrow indicates the site of injection. Panel B: Parasite biolistically bombard with beads coated with pBm5S-GFP. Panel C: Parasite microinjected with pBmtRNA-GFP. The arrow indicates the site of injection. Panel D: Non-transfected parasite maintained in culture medium and treated as above.)
produced a punctate pattern of fluorescence that localized to the hypodermal layer underlying the cuticle (Fig. 8, panel B). No fluorescence was detected in untransfected parasites maintained in parallel with the transfected worms (e.g., Fig. 8, panel D).

It is believed that the majority of all messages in the filarial nematodes contain a spliced leader (Blaxter and Liu, 1996; Maroney et al., 1995). Thus, the promoter driving the production of the spliced leader pre-RNA may be exceptionally strong. To determine if more typical promoter sequences could be studied using these transfection methods, the 499 bp located upstream of an open reading frame encoding a putative aspartyl tRNA synthetase (Kron et al., 1992) was cloned into both pGL3 Basic (designated pBmtRNA-luc) and pPD95.75 (pBmtRNA-GFP) for evaluation in the biolistic and microinjection based transfection systems. No luciferase activity was detected in isolated embryos biolistically transfected with the pBmtRNA-luc (data not shown). However, a low level of fluorescence was detected in the adult parasites microinjected with pBmtRNA-GFP. Fluorescence in the parasites injected with pBm-tRNA-GFP was confined to a small proportion of intrauterine embryos located in the vicinity of the injection site (Fig. 8, panel C).

7. Discussion

The data presented above demonstrate that the intergenic spacer of the 5S rRNA gene cluster contains a promoter capable of driving the expression of both luciferase and GFP reporter genes in transiently transfected B. malayi. Interestingly, embryos bombarded with the construct containing the O. volvulus 5S spacer produced luciferase activity levels that were similar to those produced by embryos bombarded with the homologous B. malayi construct, although little sequence similarity exists between these fragments. It is possible that the core promoter sequence recognized by the B. malayi RNA polymerase is present in both the B. malayi and O. volvulus upstream domains, but it is difficult to recognize the core promoter sequence by simple inspection of the two DNA sequences. Alternatively, the most important domains of the spliced leader promoter may reside within the spliced leader sequence itself, and/or in the short region of conserved sequence located downstream of the mature spliced leader (Hannon et al., 1990a). More experiments will be needed to differentiate these possibilities.

In adult parasites injected with pBm5S-sa-GFP, expression was detected in the intrauterine embryos proximal to the injection site. In contrast, in adult parasites biolistically transfected with the same construct, reporter gene activity seemed to be associated with the sub-cuticular tissues of the adult parasite. Microinjection introduces the exogenous DNA directly into the uterus of the adult female parasites. In contrast, microscopic analysis of the biolistically bombarded adult parasites revealed that the majority of gold particles were embedded in the cuticle or were found in the hypodermal tissue, but that none had penetrated deeply enough to enter the uterus (data not shown). It is likely that the punctate pattern of fluorescence in the biolistically transfected adult parasites results from penetration of individual gold particles into the hypodermis. Furthermore, the difference in the pattern of expression of the microinjected and biolistically transfected adults probably is a result of the deposition of the exogenous DNA in different tissues of the adult worm by the two methods.

Transient transfection may be viewed as the first step in the process of developing a stable transfection system. In this regard, it is interesting to note that the developing embryos of microinjected parasites were apparently capable of taking up exogenous DNA. By analogy to C. elegans, it is possible that the exogenous DNA in some of these embryos may form semi-stable heritable extra chromosomal arrays. If this were the case, it might be possible to isolate mature microfilaria which were transfected as embryos from microinjected adult females maintained in culture. These microfilaria might then be used to infect mosquitoes and ultimately jirds, creating a transfected parasite line. More work will be required to determine if the transfected embryos continue to develop to mature microfilaria, and if so if the exogenous DNA were stable enough to be carried through to the adult stage, and perhaps inherited to the subsequent progeny.

The studies described above demonstrate that it is possible to use particle bombardment and microinjection to transiently transflect B. malayi. The transient transfection systems described above will prove useful in studying the functional elements and regulation of B. malayi promoters and may provide means for expressing parasite proteins in a homologous system. The ability to transiently transflect B. malayi thus represents the first step in the application of reverse genetics to the study of the human filarial parasites.

Acknowledgments

We thank Drs. Bradley Yoder and Michael Bertrum for use of their microinjection and biolistic apparatuses, and Ms. Courtney J. Haycraft for assistance with the microinjection experiments. We are also grateful to Mr. Albert Towson and Mr. Shawn Williams of the UAB Imaging Facility for help in imaging the GFP experiments. Parasite material used in this project was provided by the Filariasis Repository at the University of Georgia with funds provided from the National Institute
References


