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Analysis of transcriptional regulation of tetracycline responsive genes in *Brugia malayi*

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ABSTRACT

The *Wolbachia* endosymbiont of the human filarial parasites is necessary for parasite reproduction, making it an attractive chemotherapeutic target. Previous studies have demonstrated that mRNA levels of several nuclearly encoded genes are altered as a result of exposure to antibiotics that eliminate the endosymbiont, suggesting that they may be involved in maintaining the parasite–endosymbiont relationship. Here, we tested the hypothesis that the increase in mRNA levels of certain nuclearly encoded genes of *Brugia malayi* in response to tetracycline treatment involved specific regulatory elements present in the promoters of these genes. The promoters of three such genes (BmRPL13, BmRPS4 and BmHSP70) were tested for tetracycline responsiveness utilizing a homologous transient transcription system. Reporter gene expression driven by all three promoters was up-regulated in transfected embryos exposed to tetracycline. Substitution mutagenesis was employed to map the cis-acting elements responsible for this response in the BmHSP70 promoter. Tetracycline responsiveness was found to be distinct from the cis-acting elements involved in regulating the stress response from the BmHSP70 promoter; rather, tetracycline responsiveness was mediated by a TATAA-box like element. This study represents the first demonstration of small molecule-mediated gene regulation of a native *B. malayi* promoter.

1. Introduction

The human filarial parasites are the causative agents of some of the most important neglected diseases worldwide, afflicting over 150 million individuals [1,2]. The significant morbidity associated with these infections has attracted the attention of the international community, which is currently sponsoring several programs to control and/or focally eliminate these parasites [3–5]. These programs all involve mass chemotherapy utilizing drugs that primarily affect the larval stage of these parasites (the microfilaria). As a result, in order for these programs to be successful they must maintain high drug coverage for many years to successfully control the infection [3]. This is logistically difficult to accomplish. In addition, indirect evidence suggests that resistance to ivermectin, the major chemotherapeutic agent used to treat onchocerciasis, may be emerging in some populations [6–8]. Thus, the need exists for new chemotherapeutic agents that target the adult stages of the parasite.

Approximately 30 years ago, bacteria-like structures residing within cells of the filarial parasites were first observed [9,10]. These were subsequently identified as endobacteria of the genus *Wolbachia* [11]. Experimental data suggested that these endobacteria were essential to the parasite, as treatment with certain antibiotics (including doxycycline and rifampin) that eliminated the endobacterium resulted in blockage of embryogenesis in adult female parasites [12,13] and the eventual death of the adults themselves [14]. However, the use of doxycycline or rifampin is not practical for the mass treatment of human filarial infections or two reasons. First, many of these drugs, including doxycycline are relatively toxic and are contraindicated in a large number of patient groups, including pregnant or lactating women or children. Second, clearance of the *Wolbachia* endosymbiont requires a prolonged antibiotic treatment course [15,16], something that is impractical in developing countries where the human filarial infections are endemic. For these reasons, additional research is necessary to develop new tools for control and treatment of these infections.

Abbreviations: BmHSP70, *Brugia malayi* 70 kDa heat shock protein; BmRPL, *Brugia malayi* large subunit ribosomal protein; BmRPS, *Brugia malayi* small subunit ribosomal protein; HSE, heat shock element; MFSP, major facilitator superfamily protein; nt, nucleotides; ORF, open reading frame; PCR, polymerase chain reaction; SL, spliced leader; TBP, TATAA box binding protein; *w*Bm, *Wolbachia* endosymbiont of *B. malayi*.

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We have previously hypothesized that host proteins involved in maintaining the *Wolbachia* endosymbiont might prove to be potentially important chemotherapeutic targets, since the antibiotic elimination of the endosymbiont results in parasite sterility and death. In a previous study, we began exploring this process using a *Brugia malayi* microarray to assess the effect of eliminating the *Wolbachia* endosymbiont of *B. malayi* (*w*Bm) on mRNA levels. This study demonstrated that mRNA levels of genes involved in several nuclearly encoded physiological pathways were increased in response to elimination of the *Wolbachia* endosymbiont by tetracycline treatment of adult *B. malayi*, including those involved in protein synthesis and the stress response [17]. We hypothesized that the increase in certain *B. malayi* mRNAs in response to tetracycline would involve specific regulatory elements present in the promoters of these genes.

Studies of transcriptional regulation in the human filaria have been hampered by a paucity of tools to manipulate these parasites. However, significant progress has been made employing biolisitics to transiently transfect isolated embryos of *B. malayi* [18]. Transfected embryos, although developmentally incompetent, may be maintained in culture for several days [18]. This system has been used to demonstrate that the core domains of many *B. malayi* promoters are exceptional [19–21]. In place of canonical CAAT and TATAA box elements located roughly 30 nt upstream of the start site of transcription, the core of the *B. malayi* promoters analyzed to date are localized to a pyrimidine rich region located just upstream of the spliced leader addition site [22]. Although the transient transfection system has been used extensively to study the core promoter of *B. malayi*, studies applying this method to investigate transcriptional regulatory regions in *B. malayi* promoters have been much more limited. To date, the only study investigating cis-acting promoter regulatory element has used a mutant *B. malayi* promoter containing a synthetic ecdysone response element to demonstrate that a functional ecdysone signaling pathway exists in this parasite [23]. No studies have been reported identifying endogenous cis-acting regulatory elements in *B. malayi* promoters.

In the current study, we have used the *B. malayi* transient transfection system to test the hypothesis that the increase in certain *B. malayi* mRNAs in response to tetracycline involves specific regulatory elements present in the promoters of these genes. We report evidence that this is indeed the case, and map the cis-acting element in one promoter necessary for up-regulation of transcription in response to tetracycline treatment.

2. Materials and methods

2.1. Identification of candidate genes

Candidate tetracycline-regulated genes were identified by examining an annotated list of genes whose mRNA levels were up-regulated in adult female *B. malayi* following 14 days of tetracycline treatment [17]. Preference was given to those genes encoding ribosomal proteins and/or stress proteins, as the promoters of such genes have been extensively characterized in *B. malayi* [19–22]. Based upon this analysis, three genes were chosen for further study. These included two ribosomal protein genes (BmRPS4 and BmRPL13, gene models Bm1_06615 and Bm1_47775 respectively) and the gene encoding the stress protein BmHSP70 (gene model Bm1_43675). Stable mRNA levels transcribed from these genes were increased 2.7 fold (BmRPS4), 2.36 fold (BmRPL13) and 3.30 fold (BmHSP70) in parasites treated with tetracycline [17].

To identify a promoter whose expression was not affected by tetracycline treatment, the microarray data [17] were examined to identify genes whose mRNA levels were not changed in response to tetracycline treatment *in vivo*. Genes whose stable transcript levels remained unaffected by tetracycline treatment were then ranked based on the overall level of signal detected in the microarray experiment. This was done based upon the assumption that overall signal level in the microarray was roughly proportional to the amount of the corresponding transcript in the mRNA pool, and that stable transcript levels would correspond roughly to the relative strength of the promoter of the gene from which they were derived. Based upon this analysis, the gene encoding a *B. malayi* homologue of a major facilitator superfamily protein (MFSP; gene model Bm1_38360) was chosen as a candidate whose promoter would not be affected by tetracycline treatment.

2.2. Preparation of promoter constructs

Cloning of the promoters of the BmHSP70, BmRPL13 and BmRPS4 genes have been previously described [19,21,22]. To clone the promoter of the BmMFSP gene, the region located 1194 nt upstream of the predicted open reading frame of the BmMFSP gene was amplified from *B. malayi* genomic DNA by PCR using a high fidelity DNA polymerase and the primers MFSPc (5′-GGGAACTTTACAGGAAACACAG-3′) and MFSPnc (5′-TCTCTCCTCACACTTTCTGAGAA-3′) using previously described reaction conditions [22]. The resulting amplicon was cloned into the pCR2.1 cloning vector (Invitrogen), and the DNA sequence of multiple clones determined. The amplicon from one clone whose DNA sequence was confirmed was isolated from the plasmid by digestion with *Eco*R1 and sub-cloned into the *Eco*R1 site of the renilla luciferase reporter vector pRLnull (Promega). The resulting construct was designated BmMFSP(−1194 to −1)/ren.

The series of 30 nt linker scanner mutants encompassing the BmHSP70 promoter were prepared as previously described [20]. The 10 nt and smaller substitution mutants were constructed using the Gene Tailor *in vitro* mutagenesis kit (Invitrogen) as previously described [22].

2.3. Transient transfection of B. malayi embryos and analysis of responsiveness to tetracycline

Isolated *B. malayi* embryos were transfected and promoter activity assayed by luciferase activity as previously described [19]. In brief, embryos were isolated from gravid female parasites and biolistically transfected gold beads coated with the experimental DNA. Transfected embryos were maintained in culture in the presence or absence of 40 μg ml^{-1} tetracycline for 48 h before being assayed for transgene activity. In the case of the initial experiments evaluating the response of the BmMFSP promoter to tetracycline, transfections were preformed with beads coated with the BmMFSP(−1194 to −1)/ren alone. The amount of renilla luciferase was normalized to the total protein present in each sample, which was determined using the Bradford method [24]. All subsequent assays were carried out using the dual luciferase format, in which the beads used to transfect the embryos were coated with the experimental construct driving the expression of the firefly luciferase reporter gene, and a constant amount of BmMFSP(−1194 to −1)/ren to serve as an internal control. Firefly luciferase activity was normalized to the amount of renilla luciferase activity in each sample to control for variations in transfection efficiency. Firefly/renilla luciferase activity ratios for each sample were further normalized to the activity ratio found in embryos transfected in parallel with the parental promoter cultured in the absence of tetracycline. This permitted comparisons of data collected in experiments carried out on different days. Each construct was tested in two independent experiments, with each experiment containing six transfections of each construct to be analyzed. Three of the replicate transfections from each day were cultured in the presence of 40 μg ml^{-1} tetracycline, while three