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## Core Promoter Function in *Brugia malayi*

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Core promoter function in *Brugia malayi*

by

Michelle Bailey

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Masters of Science  
Department of Global Health  
College of Public Health  
University of South Florida

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Elephantiasis

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## **Dedication**

To my mother,

Who followed her dreams to give me the opportunity to follow mine.

## **Acknowledgements**

I have been fortunate while working on this project to be surrounded by some of the most talented and innovative faculty, colleagues, staff and students in the public health field. I would like to thank Thomas R. Unnasch, Ph.D for his knowledge in molecular biology and parasitology, and also his encouragement and assistance with the experimental design of this project. I am especially grateful to Chitra Chauhan, Ph.D for her patience and guidance from start to finish. I would also like to thank Canhui Liu for his help with the transient transfection system. To everyone in the TRU lab, thank you for answering all my questions, there were a lot.

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And finally to my family and friends, your support and love is what keeps me striving to achieve all my goals in life.

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## Core Promoter Function in *Brugia malayi*

Michelle Bailey

### **Abstract**

Previous studies have indicated that the promoters of the human filarial parasite *Brugia malayi* are unusual in that they do not exhibit the CAAT or TATAA sequences usually found in the core domains of promoters of most eukaryotic organisms. Analysis of the promoters of the ribosomal proteins showed that the region flanking the splice leader (SL) addition site plays an important role in transcription and may function as the core promoter domain in *B. malayi*. To test the hypothesis that the SL addition domain is the most important essential region of the ribosomal protein promoters, the SL addition site of the BmRPL13 gene was replaced with the SL addition domains from other ribosomal protein genes from *B. malayi*. The promoter activity of the replacement constructs were tested using a transient transfection dual luciferase assay. Promoter activity with RPL13 replacement constructs was correlated with that seen in the wild type promoters, suggesting that roughly 80% of the variations seen in promoter activity among

ribosomal protein promoters is due to variation in the SL core promoter domain.



## Introduction

Lymphatic filariasis, often called elephantiasis, is a parasitic disease that raises significant concern as a global public health problem. The two most common species of lymphatic filarial parasites, *Brugia malayi* and *Wuchereria bancrofti*, put more than 1.1 billion of the population at risk of infection. Approximately 128 million people in more than 100 endemic countries are living with lymphatic filariasis. As a result of these infections more than 40 million people exhibit debilitating and disfiguring symptoms. Infections of *W. bancrofti*, transmitted by the *Anopheles*, *Culex* and *Aedes* species of mosquito, are endemic across Sub-Saharan Africa, the Pacific Islands and Latin America. *W. bancrofti* is responsible for approximately 115 million cases of lymphatic filariasis. Transmitted primarily by the *Mansonia* mosquito species, *B. malayi* is responsible for the majority of the remaining infections associated with lymphatic filariasis, and is localized to most of Southeast Asia (John & Petri, 2006; Michael & Bundy, 1997; Molyneux, Bradley, Hoerauf, Kyelem, & Taylor, 2003; Williams et al., 2000).

The filarial parasite's life cycle is quite complex, taking place in both mosquito vectors and mammalian hosts. The mosquito takes in a blood meal from an infected mammalian host, thereby ingesting the microfilaria, or first stage larvae (L1). The microfilaria undergo two molts to develop into the infective L3 larval stage within the mosquito, then migrate to the head of the mosquito to await the mosquito's next blood meal, entering the mammalian host through the bite site. The L3 larvae develop into adults in the lymphatic system of the host, finally migrating to the lymph nodes where they reside and mate (for approximately 6 years) to produce more microfilaria and perpetuate the life cycle. The parasite cannot develop into the infective stage for mammals without the mosquito and the L3 larvae cannot mature into adults without a mammalian host.

In endemic regions filarial infections are generally acquired in childhood, but symptoms do not manifest until adulthood. The symptoms of lymphatic filariasis can range from relatively mild and undetectable to severely debilitating, but are almost never fatal. Elephantiasis (swelling of the limbs, breasts and genitals) and hydrocoele (fluid-filled enlargement of the scrotum) are the result of long term chronic infections with filarial parasites. Often those infected do not exhibit any outward symptoms; however, they often have hidden lymphatic pathology and renal system damage. Although

lymphatic filariasis is generally a chronic disease, acute local inflammatory episodes in the lymph nodes and vessels can accompany chronic infections and disease manifestations (John & Petri, 2006; WHO, 2000).

The social and economic burden from lymphatic filariasis is staggering, as this disease effects primarily poor rural communities. Due to the sheer volume of infections, lymphatic filariasis is one of the leading causes of morbidity in the world. The disability that results from the more severe complications can render adults unable to work, thus putting an increased strain on the already struggling family. In addition to the economic impact there is also a severe social stigma surrounding those infected. Infected men and women that exhibit the physical manifestations of chronic lymphatic filariasis infection are viewed as undesirable mates, thus eliminating the opportunity for marriage, an essential source of security in these cultures and communities (WHO, 2002). It is not surprising that the impact this disease has on society has attracted attention and jumpstarted research and control efforts to eliminate this disease as a public health problem.

Once the physical symptoms of lymphatic filariasis manifest themselves, they cannot be reversed. Therefore, the only way for morbidity reduction is to eliminate the maturation of the microfilaria.

The ability to clear the microfilaria from the system essentially stops the transmission of the disease, because the mosquito cannot perpetuate the cycle if it cannot ingest microfilaria (Molyneux & Zagaria, 2002). Currently there are three drugs that are only effective against the microfilaria of the filarial parasite: Ivermectin, Albendazole, and Diethylcarbamazine (DEC) (Molyneux, et al., 2003). These drugs have little to no effect on the adult worms. They are, however, successful in clearing microfilaria from infected individuals when given in an oral yearly dose of DEC combined with Albendazole. In areas where onchocerciasis is endemic, Ivermectin combined with Albendazole can be used in its place to prevent the severe complications associated with DEC and onchocerciasis infections (John & Petri, 2006; Molyneux, et al., 2003).

The discovery of these successful forms of transmission prevention ultimately led to the development of The Global Programme to Eliminate Lymphatic Filariasis (GPELF) headed by the World Health Organization (WHO). With substantial financial support from the Bill and Melinda Gates Foundation, and drug donations from leading manufacturers, the program's goal is to saturate areas endemic for lymphatic filariasis with mass drug administration (MDA) over a time period sufficient for the elimination of the adult worms. (Molyneux, et al., 2003; Molyneux & Taylor, 2001; Molyneux & Zagaria, 2002)(WHO,

2000). The GPELF is currently utilizing MDA in 34 countries and has proven successful in a handful of those countries; however, drug resistance could result, thereby ending the promising works geared toward eliminating lymphatic filariasis (Molyneux & Taylor, 2001; Molyneux & Zagaria, 2002). Although no findings of drug resistance in these parasites has formally been recorded (Molyneux, et al., 2003), the history of microbial resistance necessitates caution. To combat this potential pitfall, it is extremely beneficial to study the genome of these parasites to develop new tools to fight this infection and discover targets that might represent modes of resistance. It is also beneficial to find targets that differ between the host and parasite, possibly developing treatments effective in eliminating the adult parasites. This could allow more focused treatments that would be efficient in reducing harmful side-effects on the host and eliminate prolonged treatment periods.

## Background

The Filarial Genome Project (FGP) was initiated in 1994 by the World Health Organization in an effort to augment the genomic research being done on human filarial parasites. The project's goals were to utilize a consortium of international laboratories to construct a collaborative database of genomic resources (Ghedin, Wang, Foster, & Slatko, 2004; Williams, 1999; Williams, et al., 2000). *B. malayi* was chosen as the representative model organism for this project because of its ability to be grown in a variety of animal hosts and cultured in vitro allowing all stages of life to be available for study (Denham & Fletcher, 1987; Mak, Choong, Lam, & Suresh, 1990; Nelson, Greiner, Shultz, & Rajan, 1991; Weil, Li, Liftis, & Chandrashekar, 1992). The efforts of the FGP lead to a *B. malayi* sequence database representing a nine-fold coverage of its genome (Ghedin, et al., 2004; Ghedin et al., 2007). To date, the FGP has derived 26,000 Expressed Sequence Tags (EST's) from nearly 20 cDNA libraries representing all life cycle stages of *B. malayi* and predicted approximately 11,500 genes (Blaxter, Daub, Guiliano, Parkinson, & Whitton, 2002; Williams, et al., 2000).

Even with the extensive progress that has been made to date as a result of the FGP, there is much more to be learned about the gene regulation in this organism. The lengthy life cycle of these parasites makes them impractical to study through classical genetics. Reverse genetics, a process that analyzes the function of a gene using an introduced DNA sequence, can be utilized to study gene function and regulation in the absence of classical genetic tools (Higazi, Merriweather, Shu, Davis, & Unnasch, 2002).

Previous studies have shown that that transient transfection, using both microinjection and particle bombardment are capable of driving gene expression in helminths such as *Caenorhabditis elegans*, *Ascaris suum* and Schistosome species (Davis et al., 1999; Jackstadt, Wilm, Zahner, & Hobom, 1999; Wilm, Demel, Koop, Schnabel, & Schnabel, 1999). Transient transfection is a reverse genetics technique that introduces circular DNA into an organism by either particle bombardment or microinjection. Microinjection manually introduces experimental DNA into the organism (Neuhaus & Spangenberg, 1990). Particle bombardment, a biolistics technique, propels carrier particles, such as glass, gold or tungsten, coated with naked plasmid DNA into the cells of the organism. Particle bombardment is achieved by means of gas discharge in a gun ('gene gun'), water vaporization under high-voltage electric spark or helium discharge (Jackstadt, et al., 1999;

Villemejjane & Mir, 2009). Studies have shown that microinjection is amenable to human error because it requires extensive training and skill, is more time consuming, and is therefore is a much less efficient technique (Wilm, et al., 1999). In contrast, biolistics has proven to be a rapid and simple tool for the transfection of embryos as well as multicellular organisms. Biolistics is capable of transfecting a large number of experimental organisms, produces high transformation efficiencies into a variety of cell types, and remains flexible since biophysical parameters can be adjusted to penetrate to multiple organisms and cell types (Davis, et al., 1999).

Because helminths such as *Caenorhabditis elegans*, *Ascaris suum* and Schisosome species were capable of driving gene expression in a transient transfection system, Higazi 2003 et al conducted an experiment to determine if *B. malayi* was also receptive to biolistic and microinjection techniques to study gene regulation. Higazi's experiments demonstrated that both microinjection and particle bombardment were capable of driving gene expression in transiently transfected *B. malayi* parasites. However, biolistic transfection of the embryos proved to be superior in this case because the activity was highest in biolistically transfected embryos. Microscopic analysis of the biolistically transfected adults showed that the majority of the particles did not penetrate past the cuticle.



Initially it was necessary to develop a homologous transient transfection system to study gene regulation in *B. malayi*. Rothstein and Rajan previously described the 1160nt upstream of the start codon of the *B. malayi* HSP70 gene (Rothstein & Rajan, 1991). Shu et al discovered that this region was capable of driving expression of a reporter gene to high levels in transiently transfected embryos (Shu, Katholi, Higazi, & Unnasch, 2003). It was difficult to determine which elements were driving gene expression and promoter activity in the BmHSP70, because results did not clearly reveal one region that completely reduced promoter activity. It was clear however that the CAAT and TATA boxes were not the essential regions as promoter activity did not disappear in their absence, nor was activity found in only their presence, which would have been the case if this gene contained the same regions indicative of a typical eukaryotic core promoter (Shu, et al., 2003).

Higazi 2005 et al conducted further analysis of the promoter of the HSP70 gene revealing several domains that appeared to be essential for transcription. Two of these essential domains were typical of most previously described eukaryotic promoters, such as a binding site for heat shock transcriptional factors as well as a binding site for a GAGA transcriptional factor. In contrast, the largest essential region contained the splice leader (SL) addition site, which

had never been previously shown to be an essential region of other well-characterized eukaryotic promoters. It was also reiterated that the CAAT and TATA boxes, perhaps the most universally described essential region in a typical eukaryotic core promoter, were not essential for promoter activity. As it was previously determined that promoter activity was independent from trans-splicing (Shu, et al., 2003), the data suggested that the BmHSP70 core promoter domain contains essential features that are much different from all other well-studied eukaryotic promoters(Higazi et al., 2005).

A similar analysis of the BmRPS12 gene conducted by de Olivera et al revealed three regions that when mutated resulted in a loss of greater than 90% of promoter activity, one of which was the region flanking the SL addition site (this was consistent with the previous studies on the BmHSP70 gene). When the sequences of most of the essential regions of BmHSP70 gene and BmRPS12 gene were compared there were surprisingly no striking similarities, except for the region flanking the SL addition site. This region had some degree of sequence conservation and those conserved sequences were found to be essential for activity in both the promoters. It should also be noted that the SL addition site itself in BmRPS12, when mutated, did not result in a significant loss of promoter activity consistent with the previous findings of the BmHSP70 gene. This strengthens the

assumption that the role of trans-splicing is independent of role of gene expression (de Oliveira, Katholi, & Unnasch, 2008). When taken together, both studies suggest that the region flanking the SL addition site form at least part of the essential core domain in both of these genes.

Liu et al hypothesized that the SL addition domain and its flanking sequences were necessary for transcription and served as the core promoter domain in *B. malayi* ribosomal proteins. Ribosomal proteins (RP) are constitutively expressed and were chosen for further study to reduce confounding due to upstream regulatory factors. Furthermore BmRPS12 gene, a member of the same family of proteins, was previously shown to be capable of producing activity in the transient transfection system. After a search of the EST data base, eleven ribosomal protein genes were tested in the transient transfection system, and resulted in widely variable activities, from no activity to four times the activity of BmRPS12. Six promoter sequences expressed activities that were at least 20% of the activity expressed by BmRPS12. Deletion of the 10nt upstream and 10nt downstream of the SL addition site in these six promoters resulted in a 95% reduction in reporter gene activity. This reduction in activity further supported that hypothesis that the region flanking the SL addition domain is the essential core promoter domain of *B. malayi*

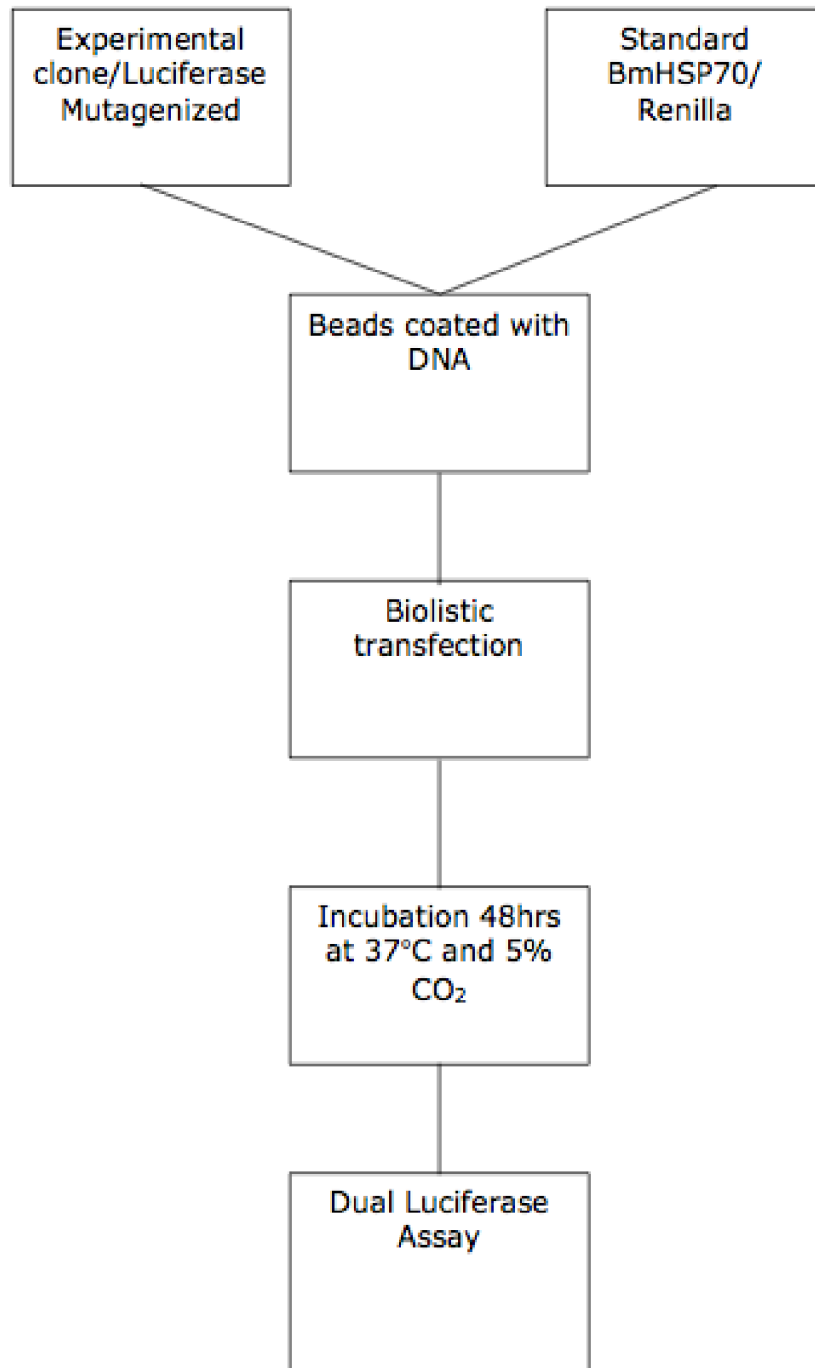
promoters. Upon alignment of the SL addition domains in all 11 RP promoters studied, it was discovered that there is a conserved pyrimidine rich motif immediately upstream of the SL addition site. The only completely conserved residue in this motif was a T at the -3 position relative to the SL acceptor site. To determine if this polypyrimidine tract was essential for activity, two mutant constructs were prepared and tested in the transient transfection assay. Mutation of the conserved T at the -3 position relative to the SL acceptor site resulted in greater than 95% reduction and the deletion of the polypyrimidine tract immediately upstream of the SL addition site resulted in greater than 90% reduction in promoter activity(Liu, Chauhan, Katholi, & Unnasch, 2009).

The data presented above from previous studies suggest that the core promoter domain lies in the region flanking the SL addition site of *B. malayi* genes. To test this hypotheses the SL addition domain of the BmRPL13 gene was replaced with the SL addition domains of other ribosomal protein genes of *B. malayi* using site directed mutagenesis and the dual luciferase transient transfection system.

## **Materials and Methods**

### *Substitution of the SL addition domains of the RP promoters*

The methodologies used in this study were followed based upon the protocol of the previous study using the transient transfection dual luciferase assay system (Figure 1)(Liu, et al., 2009). The clones used in this study were constructed during a previous study using the luciferase expression vector pGL3 Basic (Liu, et al., 2009);(de Oliveira, et al., 2008). To mutate the SL domain the GeneTailor site directed mutagenesis system was used. The primers were designed using the manufacturer's protocol. The SL addition domain flanking the SL addition site of RPL13 was replaced with the nine different RP SL addition domains of RPS 4, RPL 9, RPL 11.1, RPS 12, RPS18, RPL21, RPL 23, RPL24.e and RPL30. The 10nt upstream of the SL addition site were mutated first and the constructs derived from these mutations were then used to mutate the 10nt downstream of the SL addition site. Downstream mutations were not prepared for RPS 4, RPL 9 and RPL 23.



**Figure 1.** Flowchart representation of the methodologies used in the study.

### *Transient transfection*

Embryos from gravid adult females were isolated by dissection, transfected using biolistics, and promoter activity assayed for luciferase activity as described (Shu, et al., 2003). Plasmid DNA was mixed with a constant amount of internal standard, consisting of the BmHSP70 promoter fragment driving the expression of renilla luciferase was transfected into the isolated embryos of gravid adult females. The gold particle solution was prepared by washing the gold particles in 70% ethanol three times, followed by three washes with 100% Ethanol, and finally washed in sterile water and stored in a 50% glycerol at a concentration of 30 mg/ml at 4°C. The gold particle solution was mixed vigorously at room temperature for 15 min prior to coating with DNA. To coat the gold particles 75 µl of gold particle solution, 12µl of plasmid DNA (at 1mg/ml), calcium chloride to a final concentration of 1M, and free base spermidine (Sigma #S-3256) to a final concentration of 0.015 M were added to a 1.5 ml microcentrifuge tube while vortexing continuously. Following the addition of all components the solution was vortexed for 3 min. The DNA coated beads were then washed with 360 µl of 75% ethanol, followed by a wash with 360 µl of 100% ethanol, and finally resuspended in 30 µl of 100% ethanol. A volume of 9 µl of coated gold particles was used to bombard the embryos for each particle bombardment. The embryos

were bombarded under 1100 PSI under 20 in. of vacuum in a biolistic unit (BioRad, Hercules, CA). Approximately  $1 \times 10^{-5}$  isolated embryos were used in each particle bombardment. The parasites rested in a humid chamber for 5 minutes following bombardment and flooded with 1.6 ml of CF-RPMI tissue culture medium containing 25 mM HEPES, 20% fetal calf serum, 20 mM glucose, 24 mM sodium bicarbonate, 2.5  $\mu\text{g/ml}$  amphotericin B, 10 U/ml penicillin, 10 U/ml streptomycin, and 40  $\mu\text{g/ml}$  gentamicin. The embryos were maintained in culture tissue medium for 48hrs at 37°C and 5% CO<sub>2</sub> before being harvested by centrifugation and assayed for promoter activity.

### *Luciferase Assay*

Transiently transfected extracts were prepared and assayed for firefly and luciferase activity using the Dual Luciferase Assay System (Promega, Madison, WS). Firefly luciferase activity was normalized to the amount of renilla luciferase activity in each sample, controlling for variations in transfection efficiency. The firefly/renilla ratio was normalized further to the activity ratio of embryos transfected with the RPL13 construct that were shot in parallel in each experiment. This allowed for the comparison of data collected on different days. Each construct was transfected in triplicate and in two independent experiments carried out on different days (figure 2).



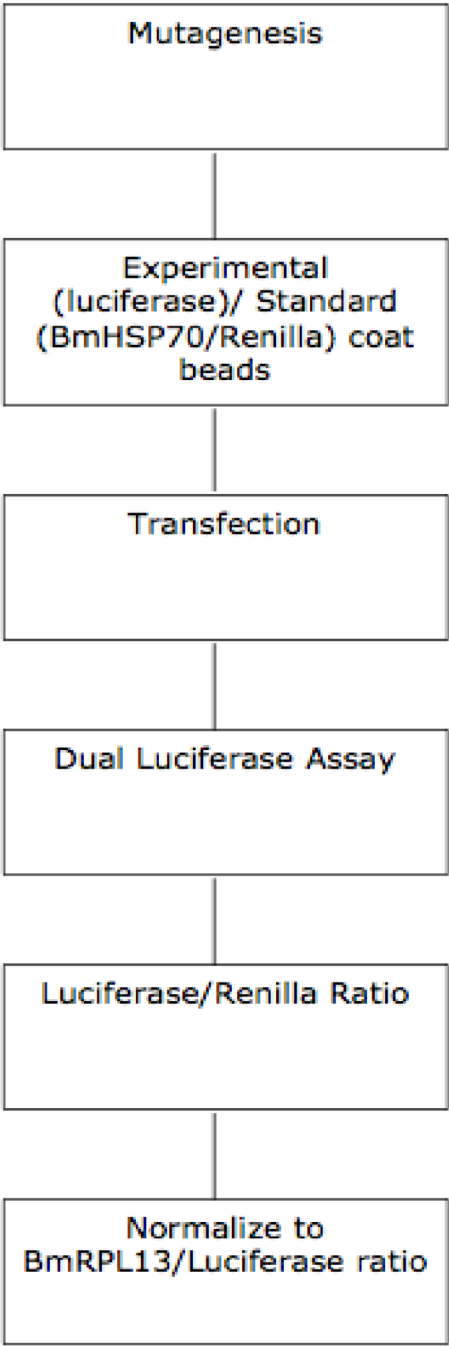


Figure 2. Flowchart representation of the Dual Luciferase Assay methodologies used in the study.

### *Statistical Analysis*

The statistical significance of the differences in activity of the native constructs and the paired experimental constructs was assessed based on the population marginal means (least squares means) using a Dunnett's test to compare the experimental constructs activities to the RPL13 control group activities. A multiple comparison analysis was performed using the Tukey method and the Tukey-Kramer adjustments to determine significant differences between the mutant promoter activities and their wild type promoter activities. All calculations were performed using the PROC GLM procedure in the SAS system, version 9.

## Results

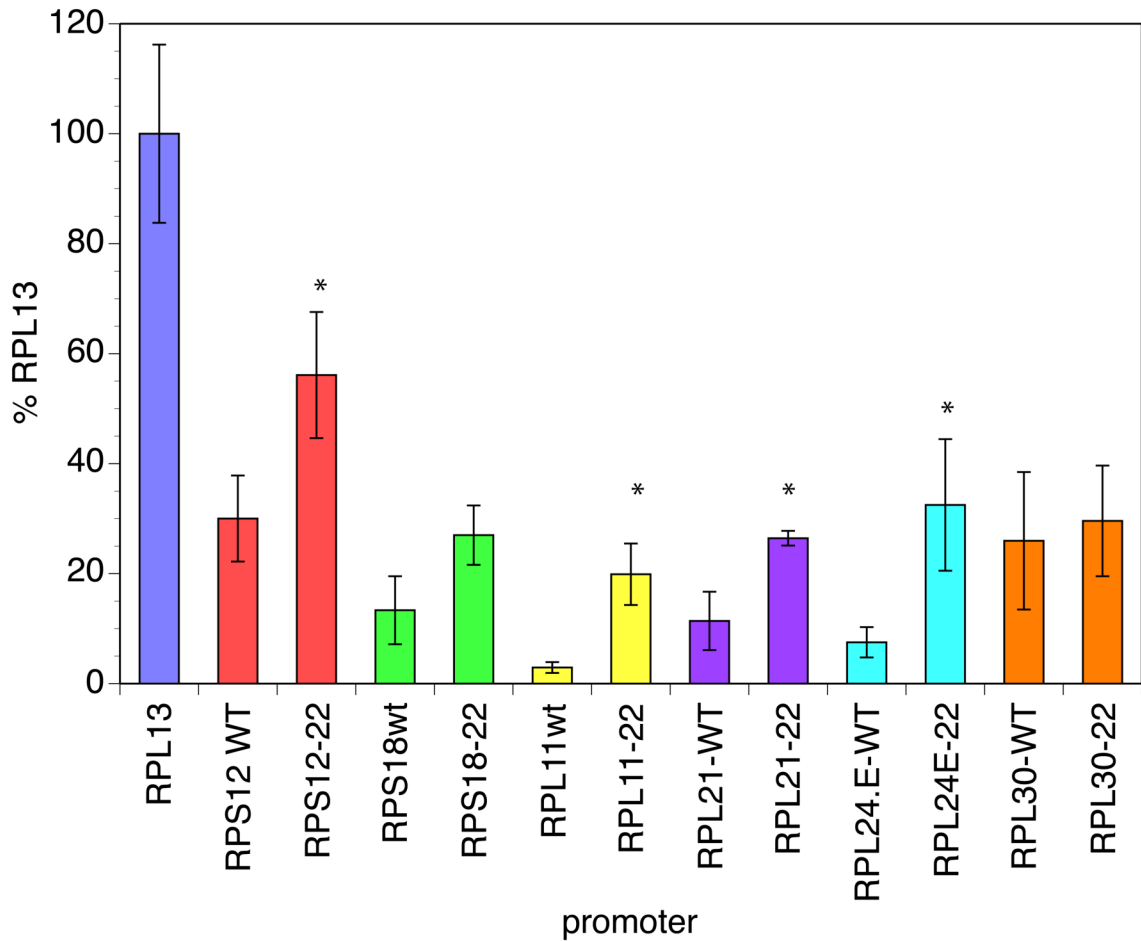
Previous studies demonstrated that several ribosomal protein promoters of *B. malayi* were capable of producing detectable levels of reporter gene activity in the transient transfection system (Liu, et al., 2009). In that study, it was hypothesized that because ribosomal protein promoters all encode proteins that are assembled in stoichiometrically equal amount in the same organelle and because that organelle (the ribosome) is constitutively expressed, the activity levels of the ribosomal protein promoters would all be roughly equal. However, experimental evaluations of the ribosomal protein promoters revealed a wide variation in their activities in the transient transfection system (Liu, et al., 2009). The goal of this study was to determine how much of this variation in promoter activity was due to polymorphisms in the putative core promoter domain and how much was due to other upstream domains. To accomplish this it was necessary to observe the reporter gene activities expressed by a number of SL addition domains in the context of an identical upstream domain. If polymorphisms in the core promoter domain were the only factor determining the variation in ribosomal promoter activity, promoter

activity should be independent of the context of the upstream sequences. If this was the case, the SL core promoter domain should give identical levels of promoter activity, regardless of the upstream domain with which it was paired. Conversely, if polymorphisms in the core promoter domain were not affecting promoter activity, all core promoter domains, when placed in the context of the same upstream domains should give identical activities. Thus, by placing the core promoter domains in identical contexts and assaying for promoter activity, it would be possible to determine how much of the activity variation seen in the ribosomal protein promoters was due to variation in the core promoters and how much was due to differing upstream domains.

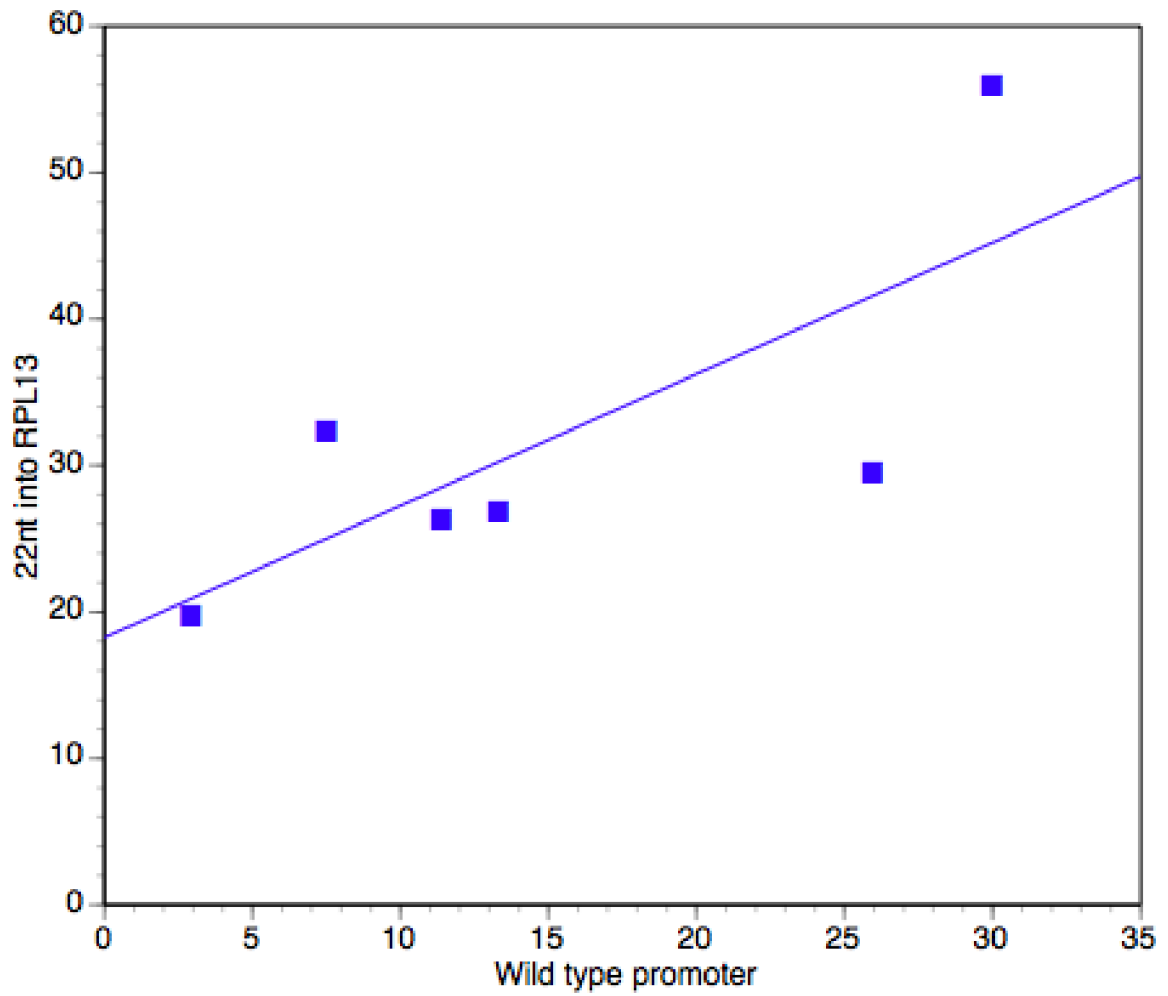
Internal replacements of the entire 22nt SL addition domain of BmRPL13 promoter region with the 22nt SL addition domain (the putative core promoter domain) of six other ribosomal proteins were prepared and tested for luciferase activity in the transient transfection system. The BmRPL13 promoter was chosen to serve as the identical upstream domain because in previous studies it exhibited reporter gene activity that was roughly 3 times that of any other ribosomal protein promoter studied (Liu, et al., 2009). Using the upstream promoter domain of the ribosomal protein with the highest native activity allowed for ease in comparison of the activities expressed by

the native BmRPL13 promoter with the activities expressed by the replacement mutants. A statistical analysis of the mean activities expressed by the native BmRPL13 promoter was compared to the mean activities expressed by the replacement mutant promoter. In all six cases, the luciferase activity expressed by the 22nt replacement mutants was significantly different from that of the native activity expressed by BmRPL13 (p-value < 0.001, dunnett's). Additionally, four of the six replacement mutants tested exhibited activity that was significantly different (p-value  $\leq$  .05) from the native gene it was derived from (Figure 3).

These data suggested that polymorphisms in the SL core domain were playing an important role in determining the variation in activity among these ribosomal promoters. To quantify the degree that the core domain polymorphisms were playing in this process, a linear regression analysis comparing the activities of the native promoter activities to the activities of the six replacement mutants described above was performed. This analysis produced a linear relationship ( $R^2=0.577$ ) with a slope of 0.90 and a y intercept of 0.18. This suggested approximately 82% of the activity expressed by the replacement mutants could be attributed to the SL addition domain and the other 18% of the activity expressed was due to upstream promoter sequences (Figure 4).



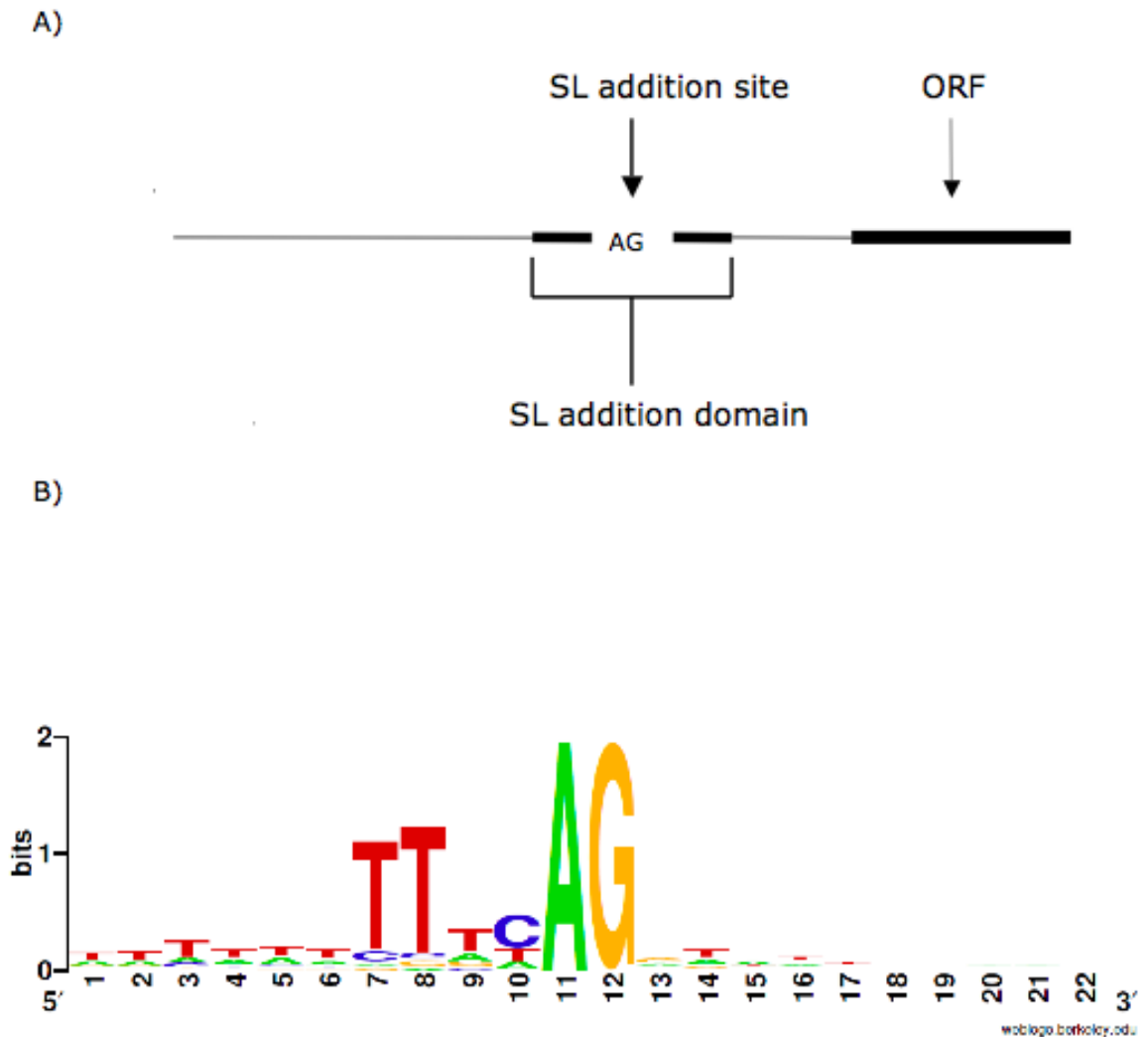
**Figure 3.** Promoter activity of native Ribosomal proteins and their 22nt replacements. Transfections and luciferase reporter assays were conducted as described in Materials and Methods. Bars represent the mean activity and the error bars represent the standard deviations of the 6 independent transfections. Colors represent the corresponding pairs of promoter constructs analyzed. Asterisks indicate replacement constructs whose activity differed significantly from the corresponding wild promoter ( $p \leq 0.05$ ; t test). All constructs exhibited activities that differed significantly from the parental BmRPL13/luc construct ( $p \leq 0.05$ ; Dunnett's test).



**Figure 4.** Correlation between native promoter activities and 22nt replacement activities. Linear regression analysis was performed and resulted in a slope of 0.90, a Y-intercept of 0.18, and an  $R^2$  value of 0.577.

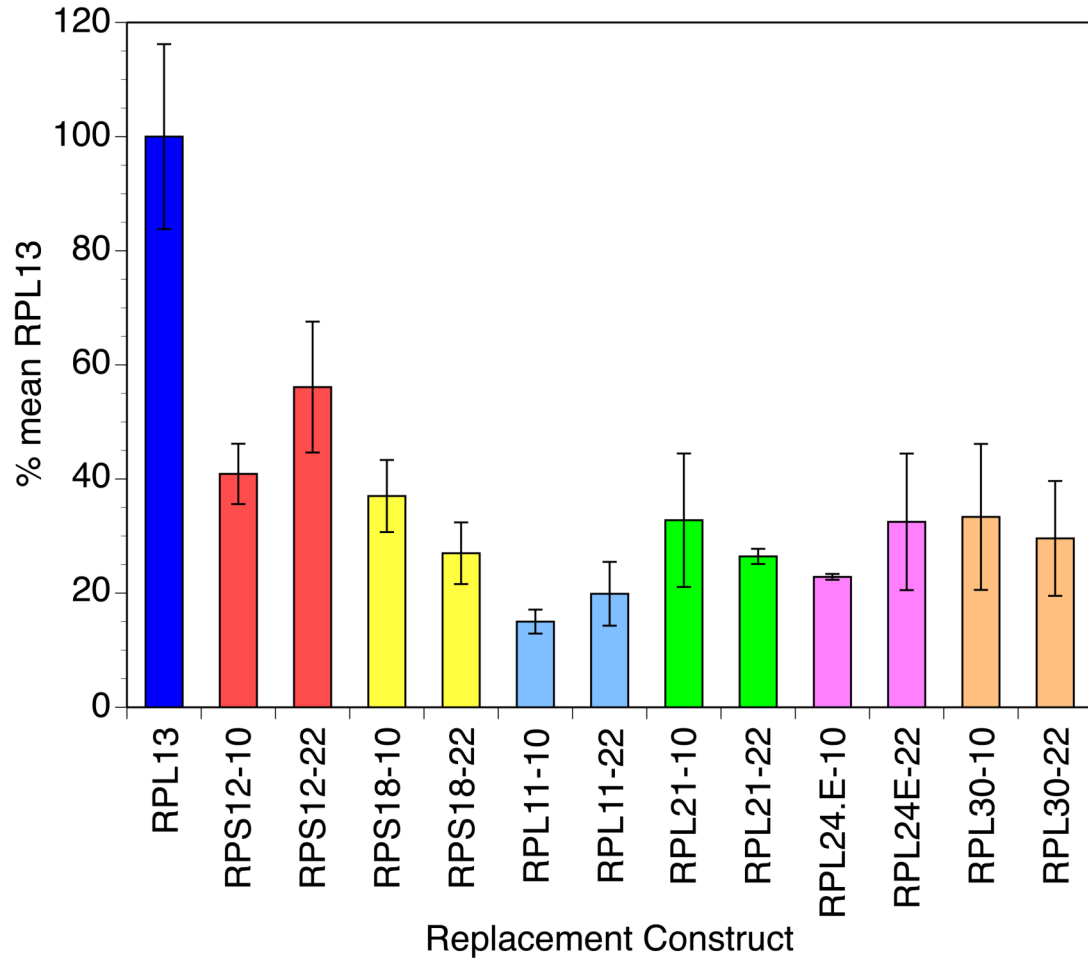
Previous studies aligning the SL addition domains of several ribosomal proteins showed that the entire domain is highly conserved. However, the 10nt upstream of the SL addition site are much more highly conserved than the 10nt downstream of the SL addition site (Figure 5). This data suggested that perhaps the 10nt upstream of the SL addition site were the most important determinants of promoter activity. The most conserved domain consisted of a polypyrimidine tract located just upstream of the SL addition domain. Mutation of this polypyrimidine tract demonstrated that it was essential for promoter activity (Liu, et al., 2009). These data suggested that within the 22nt core promoter domain, the 10nt upstream of the SL addition site were the most important in determining promoter activity. Furthermore, it could be hypothesized that polymorphisms in this 10nt upstream domain were likely to be the primary determinants in the activity variation exhibited by the different core promoter domains. To test this hypothesis, a series of internal mutations replacing the 10nt upstream of the SL addition site of BmRPL13 with those of the six other ribosomal proteins described above were prepared and tested for luciferase activity in the transient transfection system. The activities expressed by the 10nt replacement mutants were compared to the activities expressed by the 22nt replacement mutants using a t-test for statistical significance. In all cases the activities expressed by the





**Figure 5.** Alignment of the ribosomal protein SL addition domains and promoter structure of *B. malayi*. Panel A: Typical *B. malayi* promoter. The promoter region is approximately 400nt upstream of the open reading frame (ORF) and the SL addition domain in a variable distance from the ORF. This figure is not drawn to scale. Panel B: Web logo representation of the conservation within the SL addition domains of the ribosomal proteins examined.

10nt replacement mutants were statistically indistinguishable from the activities expressed by the 22nt replacements (p-value  $\geq 0.100$ ). This data suggests that the 10nt upstream of the SL addition domain are sufficient for full promoter activity to be expressed (Figure 6).



**Figure 6.** Promoter activities of the 10nt and 22nt replacements. Transfections and luciferase reporter assays were conducted as described in Materials and Methods. Bars represent the mean activity and the error bars represent the standard deviations of the 6 independent transfections. Colors represent the corresponding pairs of promoter replacement constructs that were analyzed. In all cases the activities of the 10nt replacements were not statistically different from the activities of the 22nt replacements ( $p \geq 0.100$ ; t test)

## Discussion

Previous studies on the promoter regions of *B. malayi* indicate that this parasite lacks core promoter regions, such as an essential CAAT or TATA box, typically found in most other well studied eukaryotic organisms. Instead, detailed analysis of the sequences of two other promoters, BmHSP70 and BmRPS12, suggested that the essential promoter region in this organism may be the region flanking the SL addition site (de Oliveira, et al., 2008; Higazi, et al., 2005; Shu, et al., 2003). This finding was further supported as internal deletions of the region flanking the SL addition site, in several RP promoters, resulted in a 95% reduction in activity (Liu, et al., 2009). The data from these previous studies suggested that the region flanking the SL addition site is the most essential region for promoter activity. These earlier findings made it of interest to determine whether all of the activity expressed by the ribosomal proteins of *B. malayi* could be attributed to the SL addition domain within the promoter region. The results support the previous findings that the SL addition domain is the most essential region for promoter activity. Linear regression analysis comparing the six replacement mutants to

the native activities of the promoters in which they were derived revealed that 82% of the variation in activity among the ribosomal protein promoters can be attributed to polymorphisms in the SL domain.

It is possible that like the BmHSP70 and the BmRPS12 the upstream domains of the each of the ribosomal protein genes are different. In this situation different upstream domains may have the ability to alter the level of activity expressed by these genes. As shown in linear regression analysis the upstream sequence appears to be responsible for roughly 18% of the activity expressed by these genes. To determine the validity of this finding, it would be of interest to observe the activity expressed by the ribosomal proteins when their upstream domains are placed in the context of an identical SL addition domain.

Alignment of the SL domains both in this study and in previous studies revealed a high level of sequence conservation within the SL domains of these ribosomal proteins. In addition, the 10nt immediately upstream of the SL addition site were much more highly conserved than the 10nt immediately downstream of the SL addition domain. This suggested that the more highly conserved 10nt immediately upstream of the SL addition site might be sufficient for full promoter activity. The data showed that the 10nt replacements

were capable of exhibiting the same level of promoter activity as the 22nt replacements; this suggests that the polymorphisms in the upstream domain were the primary factor in determining the relative activity of the SL core promoter domain. The 10nt upstream of the SL addition site is pyrimidine rich and the native ribosomal protein genes that exhibit the highest levels of activity have a T located at the -1 position relative to the SL addition site. Further studies to determine the particular nucleotides responsible for the variations are recommended. For example, removal of the T at the -1 position relative to the SL addition site from the genes with the highest levels of native activity or replacement of the nucleotide currently in the -1 position relative to the SL addition site with a T in the genes with lower native activity levels might reveal differences in the promoter activity following these manipulations.

Understanding the transcriptional sequences in *B. malayi* is essential for the prospect of drug discovery, the ultimate goal of the research being done on this organism. Our study and previous studies suggest the mechanism of transcription in *B. malayi* is unique. These studies suggest that the core transcriptional machinery of *B. malayi* may represent an attractive chemotherapeutic target. Currently lymphatic filariasis is being controlled by yearly mass drug administration in a number of countries to eliminate the effects of the

microfilaria on the host however; this form of treatment does not eliminate infection from the host. Thus, new drugs to treat these infections are needed if we are to eliminate them from the planet. Unique processes such as these may be instrumental in developing targeted drugs to fight lymphatic filariasis.

In addition to variations seen in the SL addition domain that explain some of the differences in promoter activity, the polymorphisms among the upstream domains may contribute to the varying degrees of activity. A detailed mapping of the upstream promoter domains of several ribosomal proteins may reveal the extent of which these sequences are contributing to the level of gene expression. Site-directed mutagenesis and series of mutations of these upstream promoter domains may reveal sequences that are necessary for control of gene expression.

To date it is still unknown what sequences or binding proteins are responsible for transcription initiation in *B. malayi*. It would be of interest to determine if any homologues of known transcription factors, such as TATA binding protein (TBP) or a TBP associated protein, are capable of interacting with the SL core promoters of *B. malayi*. Phage display, yeast one hybrid and affinity chromatography techniques might be used to screen for proteins that bind to sequences within the promoters of *B. malayi*.

Other well-characterized organisms exhibit roughly equimolar amount of the ribosomal proteins in the cell (Li, Nierras, & Warner, 1999). Thus, it is surprising that the ribosomal protein promoters exhibit such a wide variation of activity in the transient transfection system. There are two alternative explanations to explain this finding. First it is possible that the promoter activities reflect the levels of protein present, and that the ribosomal proteins in *B. malayi* are present in widely varying amounts in the stable protein pool. Alternatively, it is possible that processes downstream of transcription may be involved in determining the stable levels of the ribosomal proteins. Such processes might include differential mRNA stability, differential translational efficiency of the native mRNAs encoding each of the ribosomal proteins, or differential stability of the proteins themselves. More studies will be necessary to differentiate these alternatives. It is also possible that the level of gene expression of ribosomal proteins within this parasite varies from one life cycle stage to another. Extraction and quantitative analysis of RNA from different life cycle stages of this parasite using RT-PCR or Northern Blot may reveal differences in the level of gene expression in different stages of life.

In summary, polymorphisms in the upstream 10nt of the SL addition domain appear to play a large role in determining the strength



of promoters within this organism. Together with the data from previous studies these findings suggest that the SL addition domain represents a primary element of *B. malayi* promoters.

## References

- Blaxter, M., Daub, J., Guiliano, D., Parkinson, J., & Whitton, C. (2002). The *Brugia malayi* genome project: expressed sequence tags and gene discovery. *Trans R Soc Trop Med Hyg*, 96(1), 7-17.
- Davis, R. E., Parra, A., LoVerde, P. T., Ribeiro, E., Glorioso, G., & Hodgson, S. (1999). Transient expression of DNA and RNA in parasitic helminths by using particle bombardment. *Proc Natl Acad Sci U S A*, 96(15), 8687-8692.
- de Oliveira, A., Katholi, C. R., & Unnasch, T. R. (2008). Characterization of the promoter of the *Brugia malayi* 12kDa small subunit ribosomal protein (RPS12) gene. *Int J Parasitol*, 38(10), 1111-1119.
- Denham, D. A., & Fletcher, C. (1987). The cat infected with *Brugia pahangi* as a model of human filariasis. *Ciba Found Symp*, 127, 225-235.
- Ghedini, E., Wang, S., Foster, J. M., & Slatko, B. E. (2004). First sequenced genome of a parasitic nematode. *Trends Parasitol*, 20(4), 151-153.

- Ghedin, E., Wang, S., Spiro, D., Caler, E., Zhao, Q., Crabtree, J., et al. (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. *Science*, *317*(5845), 1756-1760.
- Higazi, T. B., Deoliveira, A., Katholi, C. R., Shu, L., Barchue, J., Lisanby, M., et al. (2005). Identification of elements essential for transcription in *Brugia malayi* promoters. *J Mol Biol*, *353*(1), 1-13.
- Higazi, T. B., Merriweather, A., Shu, L., Davis, R., & Unnasch, T. R. (2002). *Brugia malayi*: transient transfection by microinjection and particle bombardment. *Exp Parasitol*, *100*(2), 95-102.
- Jackstadt, P., Wilm, T. P., Zahner, H., & Hobom, G. (1999). Transformation of nematodes via ballistic DNA transfer. *Mol Biochem Parasitol*, *103*(2), 261-266.
- John, D. T., & Petri, W. A. j. (2006). Blood and Tissue Dwelling Nematodes. In L. Wilson & M. Hudson (Eds.), *Markell and Voge's Medical Parasitology* (ninth ed., pp. 274-321). Missouri: Saunders Elsevier.
- Liu, C., Chauhan, C., Katholi, C. R., & Unnasch, T. R. (2009). The splice leader addition domain represents an essential conserved motif for heterologous gene expression in *B. malayi*. *Mol Biochem Parasitol*, *166*(1), 15-21.

- Mak, J. W., Choong, M. F., Lam, P. L., & Suresh, K. (1990). Experimental infection of the leaf-monkeys, *Presbytis cristata* and *Presbytis melalophos* with subperiodic *Brugia malayi*. *Acta Trop*, 47(4), 223-226.
- Michael, E., & Bundy, D. A. (1997). Global mapping of lymphatic filariasis. *Parasitol Today*, 13(12), 472-476.
- Molyneux, D. H., Bradley, M., Hoerauf, A., Kyelem, D., & Taylor, M. J. (2003). Mass drug treatment for lymphatic filariasis and onchocerciasis. *Trends Parasitol*, 19(11), 516-522.
- Molyneux, D. H., & Taylor, M. J. (2001). Current status and future prospects of the Global Lymphatic Filariasis Programme. *Curr Opin Infect Dis*, 14(2), 155-159.
- Molyneux, D. H., & Zagaria, N. (2002). Lymphatic filariasis elimination: progress in global programme development. *Ann Trop Med Parasitol*, 96 Suppl 2, S15-40.
- Nelson, F. K., Greiner, D. L., Shultz, L. D., & Rajan, T. V. (1991). The immunodeficient scid mouse as a model for human lymphatic filariasis. *J Exp Med*, 173(3), 659-663.
- Neuhaus, G., & Spangenberg, G. (1990). Plant transformation by microinjection techniques. *Physiologia Plantarum*, 79(1), 213-217.

- Rothstein, N., & Rajan, T. V. (1991). Characterization of an hsp70 gene from the human filarial parasite, *Brugia malayi* (Nematoda). *Mol Biochem Parasitol*, *49*(2), 229-237.
- Shu, L., Katholi, C. R., Higazi, T., & Unnasch, T. R. (2003). Analysis of the *Brugia malayi* HSP70 promoter using a homologous transient transfection system. *Mol Biochem Parasitol*, *128*(1), 67-75.
- Villemejjane, J., & Mir, L. M. (2009). Physical methods of nucleic acid transfer: general concepts and applications. *Br J Pharmacol*, *157*(2), 207-219.
- Weil, G. J., Li, B. W., Liftis, F., & Chandrashekar, R. (1992). *Brugia malayi*: antibody responses to larval antigens in infected and immunized jirds. *Exp Parasitol*, *74*(3), 315-323.
- WHO. (2000). *Lymphatic filariasis*. Geneva.
- Williams, S. A. (1999). Deep within the filarial genome: progress of the filarial genome project. *Parasitol Today*, *15*(6), 219-224.
- Williams, S. A., Lizotte-Waniewski, M. R., Foster, J., Guiliano, D., Daub, J., Scott, A. L., et al. (2000). The filarial genome project: analysis of the nuclear, mitochondrial and endosymbiont genomes of *Brugia malayi*. *Int J Parasitol*, *30*(4), 411-419.
- Wilm, T., Demel, P., Koop, H. U., Schnabel, H., & Schnabel, R. (1999). Ballistic transformation of *Caenorhabditis elegans*. *Gene*, *229*(1-2), 31-35.

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