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Identification of Elements Essential for Transcription in *Brugia malayi* Promoters

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Little is known concerning promoter structure in the filarial parasites. Recently, transient transfection methods have been developed for the human filarial parasite *Brugia malayi*. These methods have been employed to localize the promoter for the 70 kDa heat shock protein (BmHSP70) to a region extending 394 nt upstream from the initiating codon of the BmHSP70 open reading frame. Replacement mutagenesis was used to define the elements necessary for BmHSP70 promoter activity in detail. Four domains, ranging in size from six to 22 nucleotides, were found to be necessary for full promoter activity. The two most distal domains encoded a binding site for the heat shock transcription factor and a putative binding site for the GAGA transcription factor, motifs that are found in many other HSP70 promoters. However, none of the essential domains contained sequences typical of *cis* elements that are usually found in the core domain of a eukaryotic promoter. The largest essential domain was located at positions −53 to −32, and included the splice leader addition site. These data suggest that the regulatory domains of the BmHSP70 promoter were similar to those found in other eukaryotes, but that the core promoter domain exhibited features that appeared to be distinct from those found in most other well-characterized eukaryotic promoters. An analysis of two additional promoters of *B. malayi* highly transcribed genes suggests that they also lack features commonly found in most eukaryotic core promoters, suggesting that the unique features of the BmHSP70 core promoter are not confined to this gene.

Keywords: Brugia malayi; filariasis; transcription; HSP70; transfection

Introduction

Filarial parasites represent a significant public health problem worldwide. Filaria are human parasitic nematodes, and infection with these parasites causes a number of diseases, including onchocerciasis (river blindness), elephantiasis and Loasis. It is estimated that over 140 million individuals worldwide are infected with filarial parasites, with 40 million of these suffering disfigurement or incapacitation.[1,2] Over the past decade, research efforts spearheaded by the Filarial Genome Project have resulted in a rapid accumulation of knowledge concerning the genes expressed by the human filarial parasites.[3] These efforts have culminated in the production of sequence database by The Institute for Genomic Research (TIGR) representing nine-fold coverage of the *B. malayi* genome.[4] The completion of the *B. malayi* genome sequence will open up new avenues of research into how these parasites regulate their gene expression. This will be central to understanding how this parasite has adapted to life in two very different host environments (the vertebrate and insect) and how it survives in the face of an active attack by the host immune system. With the appropriate tools, this information has the potential to link gene polymorphisms to particular phenotypes (e.g. drug resistance).

To date, little is known about how filarial parasites regulate gene expression. This is due to

Abbreviations used: RNAi, RNA interference; TBP, TATA box protein; HSE, heat shock element; HSTF, heat shock transcription factor; hHSTF, human HSTF; EMSA, electrophoretic gel mobility-shift assay; DCE, downstream core element; DPE, downstream promoter element; INR, initiator region.

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several deficiencies in the methods available to study filarial parasites. First, 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 genetic approaches can be used to study gene function, and to address key questions regarding the function and regulation of various genes. Reverse genetic approaches have proven to be extremely useful in studying promoter structure and function in the free-living nematode *Caenorhabditis elegans*. Given the primary interest in *C. elegans* as a model for development, most of the studies on promoter elements in *C. elegans* have focused on the identification of *cis*-acting regulatory elements responsible for temporal and tissue-specific regulation of gene expression. These studies have resulted in the identification of a number of upstream[5–18] and downstream[5,6,15,19] regulatory sequences responsible for both enhancing and repressing transcription from the genes in question.

In recent years, substantial progress has been made in adapting reverse genetic approaches to the study of human filarial parasites. For example, studies have demonstrated that the human filarial parasite *Brugia malayi* can be transiently transfected using both biolistic and microinjection methods.[20] RNA interference (RNAi)[21] has also been shown to be capable of reducing gene expression in both *B. malayi*[22] and in the related human filarial parasite *Onchocerca volvulus*.[23]

The HSP70 promoter is one of the most carefully studied of any eukaryotic promoter. Detailed studies from a number of organisms including *Saccharomyces cerevisiae*,[24] *Drosophila melanogaster*[25–28] and humans[29] have revealed several features that are shared among the HSP70 promoters in different organisms. First, the HSP70 gene contains a core promoter domain that extends roughly from positions −40 to +40 relative to the transcription initiation site.[26,27] The core promoter is responsible for assembling the pre-initiation complex. Formation of the pre-initiation complex is mediated through the transcription factor TF-IID, which through its TATA box protein (TBP) binding subunit recognizes the TATA box present in the core promoter domain. Further upstream of the core promoter domain, two regulatory elements are found. The heat shock elements (HSEs) consist of repeats of the sequence GAAnn repeated in alternating orientations (e.g. 5′ GAAnnTTCnnGAA 3′).[26] These elements, of which there are usually at least two, bind the heat shock transcription factor (HSTF), and result in transcription of the HSP70 mRNA in response to stress conditions. Finally, the HSP70 promoter contains a GAGA transcription factor-binding site. This factor recognizes a motif consisting of repeats of the GA-TC dinucleotide. It is a general transcription factor that is thought to be responsible for chromatin re-modeling, opening the chromatin structure to permit the binding of the proteins responsible for forming the initiation complex.[30]

Recently, our laboratory employed a homologous transient transfection system to study the promoter of the *B. malayi* 70 kDa heat shock protein homologue.[31] These studies were successful in mapping the *B. malayi* HSP70 (BmHSP70) promoter to a region extending 394 nt upstream from the start codon of the BmHSP70 open reading frame to 31 nt upstream of the start codon.[31] Analysis of the BmHSP70 promoter revealed several sequence motifs characteristic of HSP70 promoters from other organisms. These included three putative HSEs as well as putative CAAT and TATA boxes. The *B. malayi* HSP70 promoter also contained a poly purine/polypyrimidine stretch located 119–106 nt upstream of the start codon, which apparently functioned as a repressor of transcription,[31] and a spliced leader acceptor site located 47 nt upstream of the start codon. Despite these similarities to other HSP70 promoters, *in silico* analysis of the BmHSP70 promoter sequence with a number of eukaryotic promoter prediction algorithms did not result in the correct identification of the residues essential for promoter activity in the *B. malayi* HSP70 upstream domain.[31] Furthermore, deletion of the putative CAAT and TATA boxes did not result in the complete loss of promoter activity.[31] Together, these data suggest that the Bm HSP70 core promoter might exhibit features that were distinct from those of the typical eukaryotic HSP70 promoter. For this reason, we have employed the *B. malayi* homologous transient transfection system to conduct detailed linker scanner mutagenesis encompassing the entire BmHSP70 promoter. These studies suggest that the essential domains of the BmHSP70 core promoter are unique, in that they do not bear any similarity to either the TATA box or initiation region (*Inr*) domains commonly found in most eucaryotic promoters. Furthermore, a preliminary analysis of two other promoters of highly transcribed genes of *B. malayi* suggests that these also lack features commonly found in most eucaryotic promoters.

Results

Replacement mutation analysis of the *B. malayi* HSP70 promoter

Replacement mutants were prepared from a parental clone consisting of the 394 nt upstream of the start codon of the *B. malayi* HSP70 gene cloned into the luciferase reporter vector pGL3 Basic. Previous studies had demonstrated that this sequence fragment encoded the minimal promoter of the *B. malayi* HSP70 gene.[31] This clone was designated BmHSP70 (−394 to −1)/luc. A series of 14 replacement mutations (30 nt) were prepared that spanned the entire minimal promoter encoded by BmHSP70(−394 to −1)/luc. The replacement mutants were then transiently transfected into

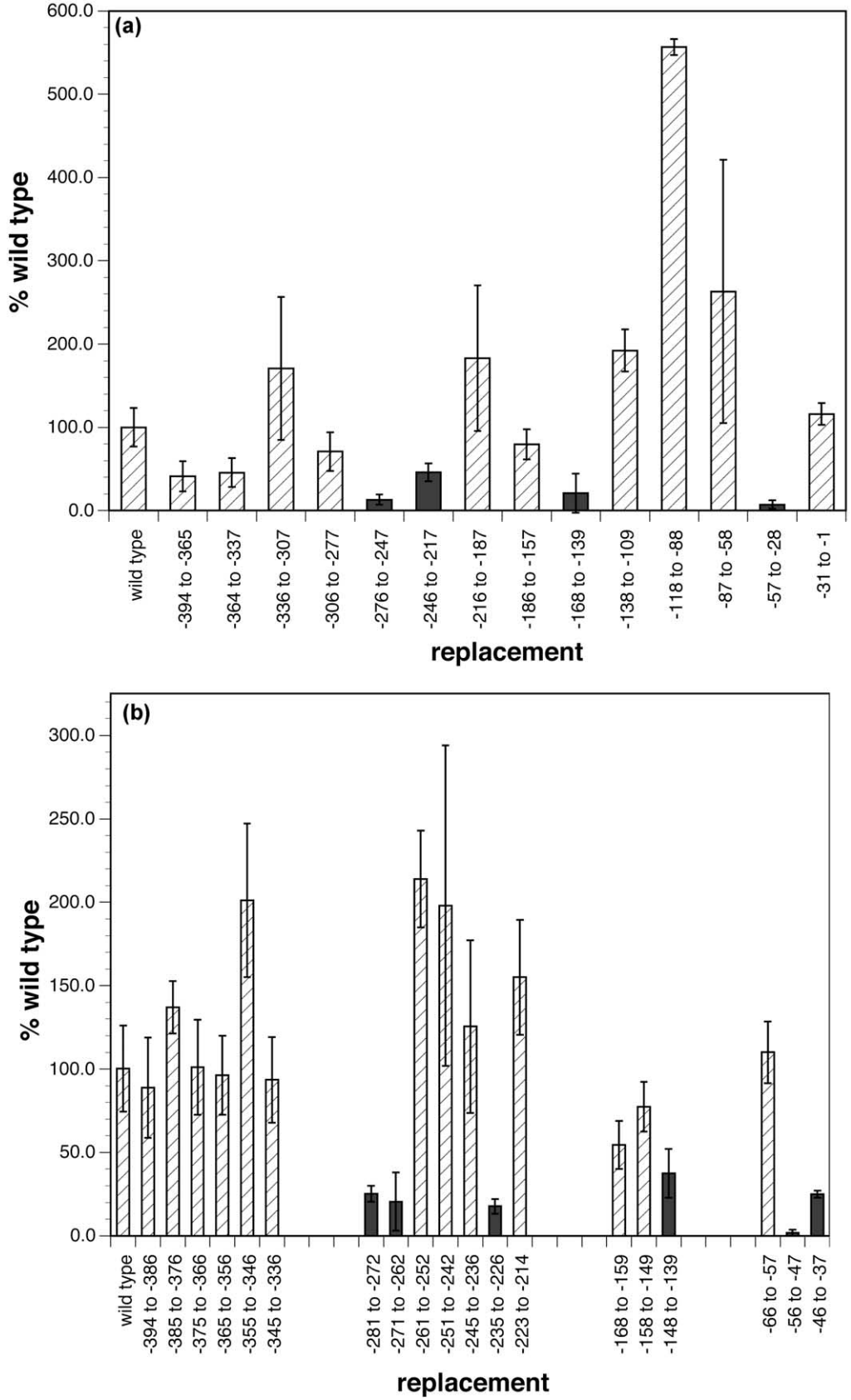

Figure 1. Block replacement mutagenesis of the BmHSP70 promoter. Replacement mutants were constructed, transfected into isolated *B. malayi* embryos and luciferase reporter gene activity assayed as described in Materials and Methods. (a) The 30 nt block replacement mutagenesis of the minimal promoter. (b) The 10 nt block replacement mutagenesis of the minimal promoter. Bars in (b) are grouped to highlight the four different domains tested on the basis of the data presented in (a). In (a) and (b) black bars indicate mutants whose activity was significantly less than that of the wild-type promoter ($p < 0.05$), while hatched bars indicate mutants whose activity was not significantly less than that of the wild-type promoter. Error bars represent the standard deviation of the activity estimates for each construct.

B. malayi embryos and luciferase reporter gene activity measured as described in Materials and Methods. The results of these studies are summarized in Figure 1. Four replacement mutants resulted in significant ($p < 0.05$) reductions in luciferase reporter gene activity, with reductions ranging from roughly twofold to over tenfold when compared to the wild-type promoter (Figure 1(a)). One substitution mutant (−118 to −88) exhibited a roughly fivefold increase in activity relative to the wild-type promoter. The −118 to −88 region contains a polypurine/polypyrimidine tract that has been shown to act as a repressor.[31] Finally, substitution mutants of the 5′-most 58 nt (−394 to −337) resulted in reporter gene activities that were roughly twofold less than those seen with the wild-type sequence. These reductions were not statistically significant. However, the standard deviations of the activity measurements of these constructs did not overlap with those of the control construct, suggesting a marginally significant effect on promoter activity.

On the basis of the results of the 30 nt substitution mutant studies, it was possible to identify four regions that encoded sequences in which substitution mutagenesis resulted in decreases in promoter activity. These included −394 to −337, −276 to −217, −168 to −139 and −57 to −28. A second series of 19 substitution mutants (10 nt) was prepared to investigate these regions in greater detail. A total of six of the 10 nt substitution mutants were found to result in significant reductions in activity relative to the wild-type promoter (Figure 1(b)). These were in and around

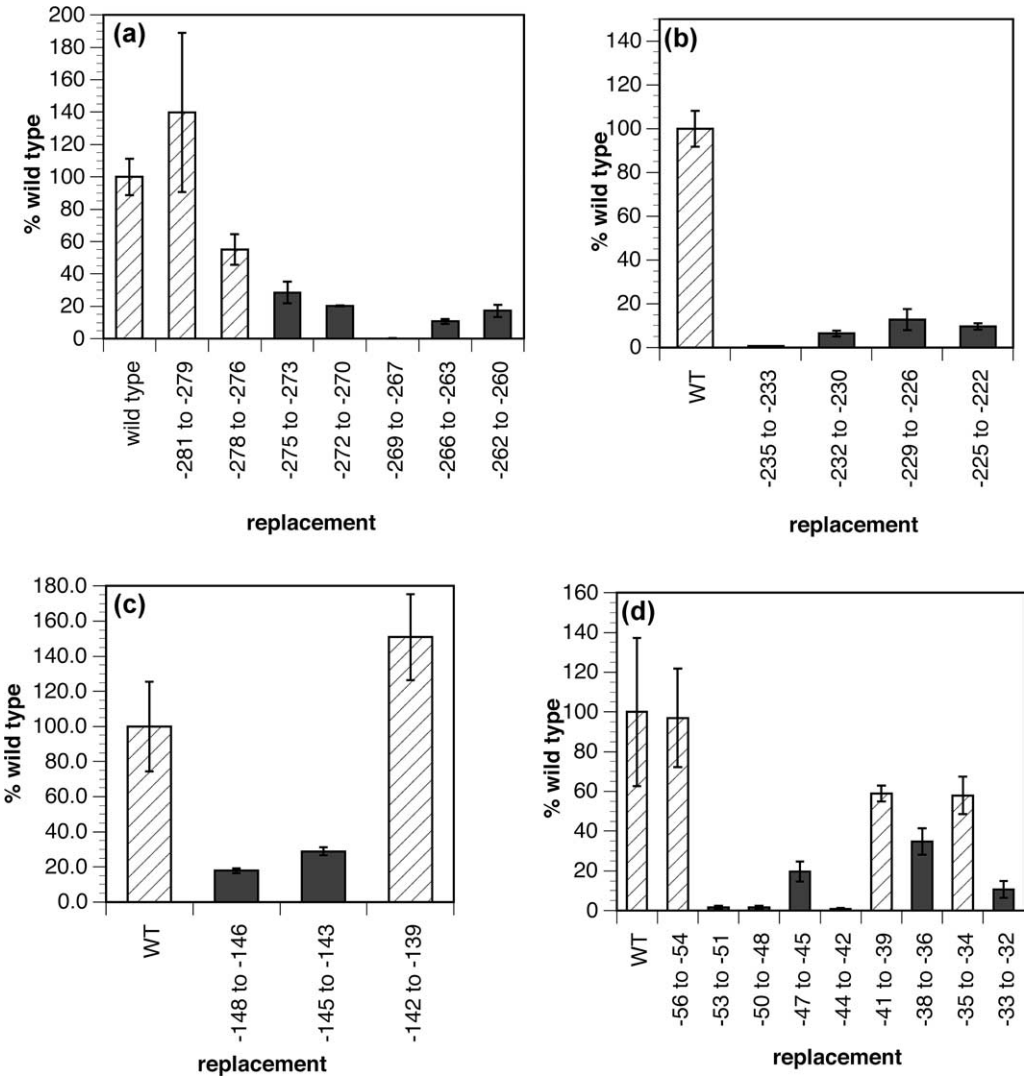

Figure 2. The 2–4 nt replacement mutagenesis of the BmHSP70 promoter. Replacement mutants were constructed, transfected into isolated *B. malayi* embryos and luciferase reporter gene activity assayed as described in Materials and Methods. (a) Analysis of the −281 to −260 region. (b) Analysis of the −235 to −222 region. (c) Analysis of the −148 to −139 region. (d) Analysis of the −56 to −32 region. In each panel, black bars indicate mutants whose activity was significantly less than that of the wild-type promoter ($p < 0.05$), while hatched bars indicate mutants whose activity was not significantly less than that of the wild-type promoter. Error bars represent the standard deviation of the activity estimates for each construct.

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      -391        -381        -371        -361        -351        -341        -331        -321        -311
CTAG AACATTCTGA CACTTCACAA TATCTCTCTC ACTCCGATGC AGTCACTCTC TCATCCTAGC CCCAATCACT TATTATCTTT

      -301        -291        -281        -271        -261        -251        -241        -231        -221
GATATTCTGC TAGCAGCACT CGCACACTGA CTATTGGAGT ACACTCTCTG CCTGTGCACT GAACATTCTG TAGTCTTCCA GAACATTCGA

      -211        -201        -191        -181        -171        -161        -151        -141        -131
TGTGTTCCAC ATATAAAGGA ACTGAGCGAT GGCTGCCTCG CTAGTGGTGA GTCGTCGTTG TACAGTGCTC GGTTGCGTTG TCGGTTGTTT

      -121        -111        -101         -91         -81         -71         -61         -51    ⇓    -41
GGGCTAGTGG AGGGGGGGGG GGGGGAAGTA GTATGCGTGG ATTGTGTGTT TGACGCTGCT TTATACGTGT GGTCTAGTTT TAGGCGTGCT
                                                                    ↑           ↑
       -31         -21         -11          +1
CGTTGTTGAT TATTGCAACG TTTCTCGAAA TACAGCAAAT ATG
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Figure 3. Regions of the BmHSP70 promoter found to be necessary for full promoter activity. Necessary domains were identified based upon the replacement mutagenesis studies shown in Figures 1 and 2. The necessary domains are indicated by grey shading. The predicted CAAT box, TATA box and the polypurine/polypyrimidine domains discussed in the text are indicated by underlining. The putative GAGA factor bonding domains are indicated by text boxed with continuous lines, and the putative HSEs by text boxed with dotted lines. The start codon is indicated by double underlining. The double arrow over the text indicates the SL addition site and the two single arrows under the text the 5′ ends of transgenic transcripts identified by 5′ RACE in a previous study.[31]

the −276 to −217, −168 to −139 and −57 to −28 regions defined by the 30 nt replacement mutants. In contrast, none of the 10 nt substitutions in the −394 to −337 domain resulted in a statistically significant decrease in activity (Figure 1(b)). On the basis of these data, it was possible to further localize the essential promoter domains to regions encompassing nucleotides −281 to −262, −235 to −226, −148 to −139 and −56 to −37. Each of these regions was then subjected to an additional round of substitution mutagenesis, in which blocks of 2–4 nt were replaced. A total of 23 such small replacement mutants were analyzed (Figure 2). Three of the four putative essential domains were found to consist of contiguous sequences with lengths that varied from 6 nt to 16 nt (Figure 2). In contrast, the largest essential domain mapped by the 30 nt and 10 nt replacements (−56 to −32) was found to contain three essential domains that varied in length from 2 nt to12 nt (Figure 2).

The essential promoter domains identified on the basis of the substitution mutant studies described above are depicted in Figure 3. As predicted by previous studies employing targeted internal deletions, neither the putative TATA or CAAT boxes were located within the domains found to be necessary for promoter activity. One of the essential domains (−235 to −222) overlapped one of three potential heat shock elements (HSEs) present in the core domain, while a second essential domain (−53 to −32) included the splice leader addition site. A third domain (the −275 to −262 region) contained a putative GAGA transcription factor-binding site. The remaining element did not coincide with any of the previously identified features of the core promoter domain.

Interaction of the putative HSEs with heat shock transcription factor

The BmHSP70 core promoter contained three putative HSEs, which were located at positions −391 to −384, −250 to −243 and −235 to −223 (Figure 3). Only one of these (−235 to −223) was found to be essential for promoter activity. HSEs activate transcription by binding heat shock

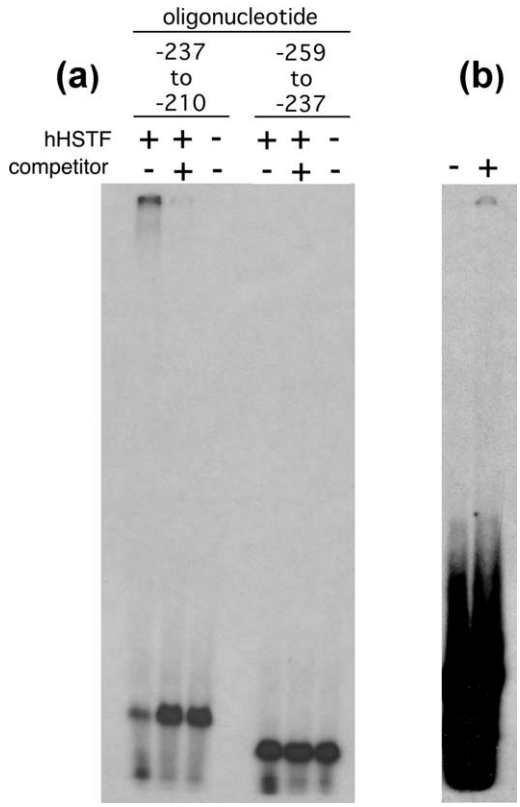

Figure 4. EMSA analysis of hHSTF binding to the BmHSP70 promoter domain. EMSAs were carried out using radioactively labeled oligonucleotides encompassing the two putative HSEs as described in Materials and Methods. (a) EMSAs carried out with purified hHSTF. Labels indicate the reagents included in each of the EMSA assays. (b) EMSA carried out with oligonucleotide −237 to −210 and crude nuclear extract from *B. malayi* embryonic cells. The lane labeled + contained nuclear extract, while the lane labeled − did not.

Table 1. Analysis of additional putative promoter domains in *B. malayi*

Cluster ID	Putative protein	% Representation in adult female datasets ($n=3333$)	% Representation in larval datasets ($n=9939$)	Human core promoter prediction	*D. melanogaster* core promoter prediction[a]
BmRBP-1	RNA-binding protein	0.75	1.3	None	−483 to −433 (0.81)
BmRPL-35	Large ribosomal subunit 35 kDa protein	0.06	0.19	None	−882 to −832 (0.96) −642 to −592 (0.95) −142 to −92 (0.82)
BmRPS-12	Small ribosomal subunit 12 kDa protein	0.39	0.10	None	−751 to −701 (0.83)
BmTub-1	α-Tubulin	0.27	0.14	None	−849 to −799 (0.90) −468 to −418 (0.84)

[a] The region identified is shown relative to the start codon of the putative open reading frame (ATG = +1). The promoter prediction score is shown in parentheses. The minimal cutoff score was set at 0.80 (the default value). The maximum score possible was 1.00.

transcription factor (HSTF). In order to determine if the BmHSP70 core promoter was capable of interacting with HSTF, electrophoretic gel mobility-shift assays (EMSAs) were conducted using oligonucleotides derived from the putative HSEs and purified recombinant human HSTF (hHSTF), as described in Materials and Methods. These studies demonstrated that hHSTF interacted with the oligonucleotide −237 to −210 (which contained the putative HSE encoded at positions −235 to −223) and that this interaction was abolished by the addition of a 100-fold excess of cold competitor oligonucleotide (Figure 4(a)). In contrast, the oligonucleotide −259 to −237 (containing the putative HSE present at positions −250 to −243) did not interact with hHSTF (Figure 4(a)). These data, when taken together, suggested that oligonucleotide −237 to −210 encoded an element capable of interaction with the *B. malayi* homologue of HSTF. In support of this hypothesis, a mobility shift was observed when an EMSA was carried out using the −237 to −210 oligonucleotide and a nuclear extract prepared from cells isolated from *B. malayi* embryos (Figure 4(b)).

Analysis of other *B. malayi* promoter sequences

The experiments described above suggested that the BmHSP70 core promoter contained elements that differed in structure from those found to exist in most eukaryotic promoters. In order to determine if this was a unique feature of the BmHSP70 promoter or something that was more generalized among *B. malayi* promoters, an analysis of the EST database was conducted. In this analysis, genes were identified for which multiple ESTs were found in the adult female EST database and which also had corresponding ESTs present in at least two larval stage databases. Over 40 such EST clusters were identified (data not shown). As expected, many of these ESTs encoded genes, such as ribosomal components and structural proteins, which would be expected to be expressed at high levels in all life-cycle stages. The cluster EST sequences were then analyzed to identify those that appeared to contain complete 5′ cDNA ends. These were then used to search the *B. malayi* genomic sequence to identify corresponding genes for which a substantial amount of upstream sequence was present. As a result of this process, four upstream domains were identified that were derived from genes that appeared to be highly expressed in multiple life-cycle stages (Table 1). The sequence domains extending 1 kb upstream of each putative start codon were then analyzed for the presence of putative eukaryotic core promoter sequences, using promoter prediction algorithms based upon data obtained from human[32] and *Drosophila melanogaster* genes†. The promoter prediction algorithm developed from human sequences did not predict a putative core promoter in any of the four upstream domains (Table 1). In contrast, the algorithm developed based upon *D. melanogaster* sequences identified a number of sequences in the upstream domains that encoded potential core promoters (Table 1).

The putative upstream domains of the genes described in Table 1 were then amplified from *B. malayi* genomic DNA, as described in Materials and Methods. PCR amplifications were successful for all of the upstream domains, with the exception of BmRPL-35 (data not shown). The amplification products were then cloned into the luciferase reporter vector pGL3 basic and tested for promoter activity as described in Materials and Methods. Both the BmRPS-12 and BmRBP-1 upstream domains exhibited promoter activity in the transient transfection system, while the BmTub-1 upstream domain did not (Figure 5).

Both the BmRPS-12 and BmRBP-1 upstream domains contained regions that were identified as potential core promoter domains by the *D. melanogaster* trained algorithm. To determine if these putative core promoter domains were necessary for promoter activity 5′ deletions were prepared that removed these putative core promoter domains. Deletion of the putative core promoter domain of the BmRPS-12 upstream region (construct BmRPS-12 (−688 to −1)/luc) had no effect on promoter activity (Figure 6(a)). In contrast, deletion of the upstream region containing the

† www.fruitfly.org/cgi-bin/seq_tools/promoter.pl

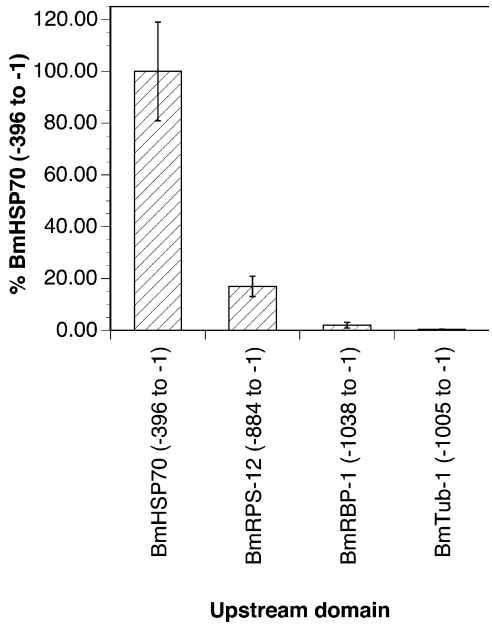

Figure 5. Promoter activity of upstream domains of genes that are expressed in multiple *B. malayi* life cycle stages. Upstream domains were obtained from a number of highly expressed genes and tested for promoter activity as described in the text. Error bars represent the standard deviation of the activity estimates for each construct.

predicted core promoter of the BmRBP-1 gene (construct BmRBP-1(−431 to −1)/luc) resulted in a loss of roughly 80% of promoter activity (Figure 6(b)). To determine if this loss in activity was indeed due to deletion of the predicted core promoter, an internal deletion was prepared in which only the predicted core promoter domain was deleted from the BmRBP1 upstream domain (construct BmRBP-1(−1038 to −1;▽ −483 to −430)/luc). Luciferase gene reporter activity from embryos transfected with this construct were not statistically different from that seen in embryos transfected with the entire BmRBP-1 upstream domain (Figure 6(b)), suggesting that the predicted core promoter domain was not necessary for promoter activity.

Given that the predicted core promoter sequences in both BmRBP-1 and BmRPS-12 were not essential for promoter activity, it was of interest to determine if these promoters had any features in common with the essential domains of the BmHSP70 promoter. From the data presented above, it could be hypothesized that the −53 to −32 domain of the BmHSP70 promoter represented the major segment of the core domain, which might also include the essential motif located at positions −148 to −143. As indicated in Figure 3, the −53 to −32 domain encompassed the SL addition site. Alignment of the sequences surrounding the SL addition sites in BmRBP-1 and BmRPS-12 upstream domains with the −53 to −32 domain of the BmHSP70 promoter revealed no conserved region that was present in all

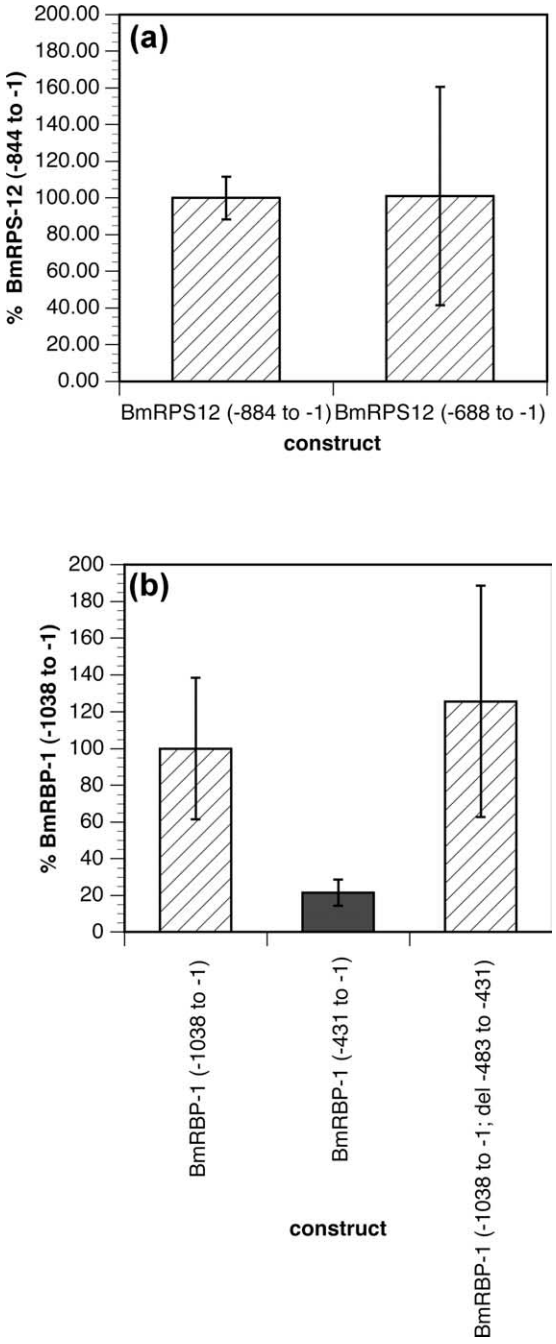

Figure 6. Deletion analysis of the putative core domains of BmRPS-12 and BmRBP-1. The 5′ deletions of the upstream domains of BmRPS-12 and BmRBP-1 and a targeted internal deletion of the putative core domain of BmRBP-1 were prepared as described in the text. Black bars indicate mutants whose activity was significantly less than that from the wild-type promoter ($p < 0.05$), while hatched bars indicate mutants whose activity was not significantly less than that of the wild-type promoter. Error bars represent the standard deviation of the activity estimates for each construct. (a) The effect of 5′ deletion of the putative core domain of BmRPS-12 on promoter activity. (b) The effect of 5′ and targeted internal deletions of the putative core domain of BmRBP-1 on promoter activity.

Figure 7. Motifs similar to the −53 to −32 essential domain of the BmHSP70 promoter in the BmRBP-1 and BmRPS-12 promoters. The essential nucleotides in the BmHSP70 −53 to −32 domain are indicated by grey shading. Nucleotides identical with those in the BmHSP70 −53 to −32 domain in the BmRBP-1 and BmRPS-12 promoters are indicated by dots. The coordinates in each promoter refer to the distance upstream of the initiating codon, as all three native mRNAs derived from these genes are *trans* spliced.

three promoters (data not shown). When a more global search was conducted of the BmRPS-12 and BmRBP-1 upstream domains, both promoters were found to contain a region that exhibited >75% sequence similarity with the essential residues in the −53 to −32 region of the BmHSP70 promoter (Figure 7). However, neither promoter contained a sequence within 100 nt upstream of these elements that is greater than 60% identical with the −148 to −143 domain of the BmHSP70 promoter (data not shown).

Discussion

The experiments described above have resulted in the identification of five motifs in the BmHSP70 promoter that are involved in promoter activity. Replacement of four of these led to a decrease in promoter activity, while the replacement of one (containing the polypurine domain) led to a significant increase promoter activity.

There are two putative HSEs present in the roughly 280 nt upstream of the HSP70 start codon that are necessary for full promoter activity. Previously, we have demonstrated that luciferase activity in embryos transfected with the construct containing the *B. malayi* HSP70 promoter driving a luciferase reporter gene was not increased in embryos exposed to heat shock.[31] This is surprising, given the fact that the data provided above suggests that replacement of one of the putative HSEs in the *B. malayi* HSP70 promoter results in an approximately 90% reduction in luciferase activity (Figure 2(b)). Futhermore, studies of the expression of the endogenous *B. malayi* HSP70 gene by Northern blot analysis demonstrated that expression of this gene is upregulated in intact parasites in response to heat shock.[33] We hypothesize that the reason for the lack of upregulation in response to heat shock in the transiently transfected embryos is a result of the fact that upregulation of HSP70 expression can be induced by a number of factors in addition to heat shock. The transient transfection system used here relies upon biolistically transfected embryos that are dissected from the female's uterus. These embryos do not continue to develop in culture and can be maintained for roughly one week when removed from the uterus of the adult female. It is likely that removal of the embryos from the uterus of the adult female results in enough stress to fully up-regulate HSP70 transcription, so that no up-regulation in response to additional stress imposed by heat shock was seen.[31] This hypothesis would explain the high level of expression that we observe from embryos transfected with constructs containing the HSP70 promoter when compared to the other two promoters included in this study (BmRPS-12 and BmRBP-1) and why the HSE was necessary to obtain this high level of BmHSP-70 driven-expression in transfected embryos. In this context, it should be noted that both of the latter promoters were found to be quite active in the assay, producing luciferase levels that were at least 100 times greater than background (data not shown). However, the BmHSP-70 promoter is extremely active in the transiently transfected embryos, probably due to the stress response induced in these embryos when they are placed in culture as described above.

The finding that the minimal *B. malayi* HSP70 promoter contained two putative HSEs is similar to what is found in other eukaryotes, where multiple HSEs are commonly found upstream of the core promoter. Interestingly, however, replacement of only one of these two putative HSEs resulted in a significant reduction of promoter activity. Furthermore, EMSA demonstrated that only the essential HSE was capable of interacting with hHSTF. However, in *D. melanogaster*, the HSEs appear to act cooperatively in upregulating transcription.[26] It is possible, therefore, that while the HSE at −235 to −223 serves as the primary nucleation site for HSTF, the HSE at −251 to −242 may act cooperatively in binding additional heat shock transcription factor once the site at −235 to −223 is filled, but cannot interact with HSTF in the absence of the primary domain.

The discovery of HSEs in the minimal promoter of the BmHSP70 gene is in accord with what is known concerning the regulation of heat shock family genes in the free-living nematode *Caenorhabditis elegans*. A recent bioinformatic study characterizing putative regulatory elements in the upstream regions of genes up-regulated in response to heat shock in *C. elegans* revealed that such genes contain a sequence motif characteristic of the canonical HSE.[12] The role of one such element in a heat shock responsive gene (the *hsp-16-2* gene) was confirmed experimentally. This analysis identified a second regulatory motif (designated the heat shock-associated site or HSAS) whose role in regulating transcription from the *hsp-16-2* gene was also confirmed experimentally.[12] In another experimental study, an additional regulatory element in the *C. elegans hsp-16* gene was identified recently that was responsible for regulation of gene expression in response to hypoxia but not to other forms of stress.[34] However, motifs similar to neither the HSAS or hypoxia regulatory element are present

in the essential domains of the BmHSP70 promoter (data not shown).

In addition to the HSEs, the *D. melanogaster* promoter contains sequences that bind the GAGA transcription factor. The GAGA transcription factor recognizes a sequence consisting of a number of repeats of the dinucleotide (CT/GA). The GAGA transcription factor is thought to be involved in chromatin remodeling, permitting access of the transcription factors involved in formation of the initiation complex to the core promoter.[30] There are three potential GAGA transcription factor-binding sites in the *B. malayi* HSP70 promoter: at positions −367 to −361, −345 to −339 and −267 to −262. One of these (−267 to −262) is contained within one of the regions found to be necessary for activity.

In all other eukaryotic HSP70 promoters characterized to date, the promoter contains an easily identifiable core domain anchored by a TATA box, which is essential for promoter activity.[35] However, as this study demonstrated, replacement of the only putative TATA box present in the BmHSP70 promoter did not result in a significant reduction in promoter activity. This result is in accord with our earlier work, which demonstrated that a construct in which the putative TATA box was deleted still exhibited roughly 50% of the activity of the wild-type promoter, which suggested also that the putative TATA box was not essential for promoter activity.[31] In the current study, the 30 nt replacement mutant encompassing the putative TATA box exhibited an activity that was nearly identical with that of the wild-type construct (Figure 1(a)). A statistical analysis comparing the relative activity of this 30 nt replacement to that obtained from TATA box deletion reported in the previous study demonstrated that the difference in the activity produced by the two constructs was not statistically significant ($p=0.128$). Taken together, these data suggest that the putative TATA box is not involved in transcription from this promoter.

There are many examples of TATA-less promoters from other organisms, but the mechanism of transcriptional initiation from such promoters is incompletely understood. In both *D. melanogaster*[36,37] and humans,[38] sequences located approximately 30 nt downstream of the transcriptional start site can serve as a site for TFIID complex binding. These sites are termed downstream core elements (DCEs),[38] or downstream promoter elements (DPEs).[37] In this regard, it is interesting to note that the largest essential domain mapped by the work described here is located at positions −53 to −32, which encompasses the acceptor site for the spliced leader. The essential activity of this region is not associated directly with the *trans* splicing process, as previous studies have demonstrated that mRNAs transcribed from transgenes containing the BmHSP70 upstream domain alone are not *trans* spliced.[31,39] The −53 to −32 domain is roughly 30 nt downstream from the two 5′ ends of the transgenic mRNA mapped by 5′ RACE in previous studies (Figure 3).[31] This region also contains a sequence (AGGCGTG) that is closely related to the consensus DPE of *D. melanogaster* (RGWCGTG; R=A or G, W=A or T). Finally, detailed mapping of the −53 to −32 region identified three sub-domains that were necessary for full promoter activity (Figures 2 and 3). This finding is similar to the DCE encoded in the human β-globin promoter, which also consists of three sub-domains.[38] Thus, it is tempting to speculate that the −53 to −32 domain encodes a promoter element that functions in a fashion analogous to the DPE of *D. melanogaster* or the DCE of humans. However, if this is the case, the −53 to −32 domain exhibits some unique properties. For example, the human DCE is functional only in the context of a TATA box, which is lacking in the *B. malayi* HSP70 promoter. Similarly, the *D. melanogaster* DPE is only functional in the context of a functional initiator region (INR), a sequence that encodes the start site of transcription.[37] Furthermore, spacing between the INR and the DPE is critical for DPE functionality.[37] However, positions −100 to −61 in the *B. malayi* HSP70 promoter (which is where one might predict the INR to be found if −53 to −32 was functioning as a DPE) are not required from promoter activity, and this region does not contain any homology to the consensus sequences for either the *D. melanogaster* or mammalian INRs (data not shown).

Examination of the −53 to −32 domain does not reveal any sequence that exhibits significant homology to TBP-binding sites or any other components of the TFIID complex. The only transcription factor-binding site predicted to be present in the −53 to −32 region is for p53, a mammalian tumor suppressor that acts as a transcriptional repressor on TATA box-containing mammalian promoters.[40] As these studies have demonstrated that the *B. malayi* HSP70 promoter does not contain a functional TATA box, it is unlikely that this domain encodes a binding site for a *B. malayi* p53 homologue.

The studies of the other promoters described above, suggest that the lack of motifs that define most eukaryotic core elements is not a property that is unique to the BmHSP70 promoter. An analysis of four other upstream domains from genes that are expressed in multiple life-cycle stages of *B. malayi* revealed that none of these putative promoter domains contained a sequence that was predicted to be core promoter elements by an algorithm trained on human promoter sequences. Analysis of these putative promoter domains using a program trained on *D. melanogaster* promoters identified several sequences that might represent putative core promoter domains. However, deletion of these putative core domains from two of these promoters (BmRPS-12 and BmRBP-1) did not result in a reduction in promoter activity. Together, these data suggest that the unique features of the BmHSP70 promoter are not specific to this particular promoter, but may be found in other *B. malayi* promoters as well. These data further suggest that promoter prediction algorithms that have been

trained on sequences from other eukaryotes may not be effective in accurately identifying promoters in *B. malayi*.

Other published studies have suggested that the lack of an identifiable TATA and CAAT box may be relatively common among the promoters of both parasitic and free-living nematodes. Surprisingly little is known concerning the structure of the core promoter domains in *C. elegans*, as the majority of the promoter structural studies in this organism have concentrated upon the identification of *cis*-acting elements responsible for regulating tissue and developmental expression. However, in the studies that have examined the upstream domains of *C. elegans* genes for core promoter elements, most have not succeeded in identifying TATA or CAAT boxes. For example, TATA boxes have not been found in the *ace-1* gene,[14] the *unc54* myosin heavy chain gene,[41] or in the *myo1* or *myo2* genes[42] of *C. elegans*. A putative TATA box has been identified in the *hsp-16-2* gene cluster;[43,44] however, the role of this putative TATA box in transcription has not been confirmed experimentally. Similarly, TATA boxes were not found in the upstream domain of the *alt1* gene or the *alt 2* gene of *B. malayi*,[45] or in the Ov*sod1*, Ov*sod2*, Ov*GST-1a* and Ov*GST-1b* genes of the closely related human filarial parasite *O. volvulus*.[46,47] All of these upstream domains were capable of driving transcription when transfected into *C. elegans*.[45–47] Taken together, these data suggest that the lack of canonical CAAT and TATA boxes may be a common feature of nematode promoters in general.

In summary, the results of this study revealed that the BmHSP70 promoter shares some common functional regulatory domains with the HSP70 domains of other organisms, including a functional GAGA factor-binding site and a functional HSE. However, this promoter lacked functional sequences typical of those found in most eukaryotic core promoters. This finding was not unique to the BmHSP70 promoter, as an analysis of two additional promoters revealed a similar lack of a conserved core promoter domain. Further studies will therefore be necessary to define what makes up a core promoter in *B. malayi*. These studies will require detailed mapping of additional promoter sequences, as well as the accurate determination of the start sites of transcription, and an analysis of the transcription factors that bind to the essential domains.

Materials and Methods

Construction of replacement mutants and promoter isolation

Three different classes of BmHSP70 replacement mutants were used in this study. The first consisted of a series of 30 nt replacements that spanned the entire 394 nt upstream domain of the start codon of the BmHSP70 gene. The clone BmHSP70 (−394 to −1)/luc served as the parental plasmid for constructing these mutants. This clone consists of the 394 nt upstream of the start codon of the BmHSP70 ORF cloned into the luciferase reporter vector pGL3 Basic (Promega, Madison, WI). The 30 nt replacements were constructed using a modification of the targeted deletion strategy described by Shu *et al*.[31] In brief, the parental plasmid was used as a template in an inverse PCR reaction together with outward-pointing primers that were designed to span 30 nt gaps in the HSP70 promoter sequence. The amplification reactions were carried out using a high-fidelity thermostable DNA polymerase (Turbo *Pfu*, Stratagene, La Jolla, CA). The inverse PCR amplification primers contained synthetic SpeI sites at their 5′ ends to facilitate subsequent recovery of mutants. Following PCR amplification, the resulting amplicons were digested with SpeI and gel-purified, as described.[31] The purified products were self-ligated and transformed into *Escherichia coli* DH5α competent cells. Individual colonies were recovered, and their DNA sequence determined. Plasmid DNA was then recovered from a culture of a clone, the sequence of which was confirmed, and digested with SpeI. The digested plasmid was then ligated to double-strand linker with the sequence 5′ pCTAGTTTTACACTCGAGCCAATTA 3′. This oligonucleotide was an irrelevant stuffer fragment derived from the tail component 144 gene of the bacteriophage lambda, which was modified to contain an internal Xho1 site and SpeI sticky ends. Following linker ligation, the ligation products were digested with Xho1 (to eliminate multimeric ligation of the stuffer), re-ligated and transformed into *E. coli* as described above. Representative clones were then isolated and their DNA sequence confirmed. This process resulted in the construction of 14 replacement mutants (30 nt) that together spanned the entire BmHSP70 core promoter domain. In each of these mutants, the target 30 nt sequence was replaced by the sequence 5′ ACTAGTTTTACACTCGAG CCAATTACTAGT 3′.

A series of 20 replacement mutants (10 nt) was constructed that targeted the essential domains identified on the basis of the data obtained from the 30 nt replacement studies. The 10 nt replacements were constructed using an inverse PCR protocol similar to that described above. In this case, the BmHSP70 (−394 to −1)/luc parental plasmid was employed as a template in inverse PCR amplification reactions that included primers that spanned defined 10 nt gaps in the promoter domain. The primers contained synthetic SpeI sites followed by two irrelevant nucleotides at their 5′ ends. Amplicons resulting from the amplification reactions were digested with SpeI, gel-purified and self-ligated, as described above. Selected clones were isolated and their DNA sequences determined to confirm that the amplification process had not introduced any unwanted mutations. This process resulted in the replacement of defined 10 nt regions of the promoter domain with the sequence 5′ NNACTAGTNN 3′, The two 5′ and the two 3′ nucleotides differed from construct to construct, depending upon the sequence that was replaced.

Finally, a series of 22 triplet replacement mutants were constructed based upon the results obtained from the experiments utilizing the 10 nt replacement mutants. Triplet mutants were prepared using the parental plasmid BmHSP70 (−394 to −1)/luc and mutated oligonucleotide primers with the GeneTailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. In preparing the triplet mutants, each base to be mutated was replaced by the one that was most distantly related to it. Thus, A was replaced with C,

G with T, C with A and T with G. As before, the DNA sequence of all clones was determined to confirm that they contained the desired mutation and no other.

The upstream domains of the BmRPS-12, BmRPL-35, BmTub-1 and BmRBP-1 genes were identified by comparison of the cluster EST sequences to the genomic DNA sequence produced by The Institute for Genomic Research†.[4] Primers designed for PCR amplification of the approximately 1000 nt upstream of the start codon in each of these genes were used to amplify the corresponding sequences from *B. malayi* genomic DNA, as described.[20] The amplification products were cloned into the original TA cloning vector, as described,[20] and the DNA sequence of selected clones confirmed. The inserts of clones whose sequences were confirmed were then excised with EcoRI and cloned into the luciferase reporter vector pGl3 basic, as described.[31] Targeted deletions of the promoters were prepared in the pGL3 basic sub clones, using the SpeI-mediated inverse PCR protocol described above.

Transient transfection and analysis of promoter activity

Isolated *B. malayi* embryos were transfected and promoter activity assayed by luciferase activity as described.[31] In brief, embryos were isolated from gravid female parasites and transfected with the experimental DNA driving the expression of firefly luciferase mixed with a constant amount of an internal standard, consisting of the BmHSP70 promoter fragment (positions −659 to −1) driving the expression of renilla luciferase (construct BmHSP70 (−659 to −1)/ren).[31] Transfected embryos were maintained in culture for 48 h before being assayed for transgene activity. Firefly luciferase activity was normalized to the amount of renilla luciferase activity in each sample to control for variations in transfection efficiency. The ratio of firefly activity to renilla activity for each sample was further normalized to the activity ratio found in embryos transfected in parallel in each experiment with the parental construct (BmHSP70 (−394 to −1)/luc). This permitted comparisons of data collected in experiments carried out on different days. All constructs were assayed at least in triplicate.

The statistical significance of differences in the activity of the experimental constructs was determined using Dunnett's test, as described.[31] In conducting the statistical analysis, the data from each construct were compared to the activity detected in embryos transfected with the wild-type construct in parallel on the same day.

Preparation of nuclear extracts from *B. malayi* embryos

B. malayi embryos were isolated by dissection of 100 gravid adult females and cells were released from embryos as described.[48] Nuclear extracts were then prepared from the cells using a commercially available kit (Active Motif, Carlsbad CA), following the manufacturer's protocol. In brief, the isolated cells were washed in ice-cold PBS supplemented with phosphatase inhibitors (Active Motif). The pelleted cells were resuspended in 500 µl of hypotonic buffer (Active Motif) and incubated on ice for 15 min. Cells were then treated with 25 µl of detergent solution (Active Motif) and vortex mixed for 10 s followed by centrifugation for 3 min at 14,000*g* at 4 °C to pellet the nuclei. The nuclear pellet was resuspended in 50 µl of lysis buffer supplemented by 1 mM DTT and protease inhibitors (Active Motif), vortex mixed for 10 s and incubated on ice on a rocking platform for 30 min. The extract was then subjected to centrifugation at 14,000*g* at 4 °C for 10 min. The supernatant (the nuclear extract) was stored at −80 °C.

Electrophoretic mobility-shift assays (EMSA)

EMSAs were carried out essentially as described.[34] In brief, complementary oligonucleotides corresponding to positions −237 to −210 and −259 to −237 were synthesized and 5′ end-labeled with ^{32}P using [^{32}P]ATP and bacteriophage T4 polynucleotide kinase. Equal amounts of the labeled complementary oligonucleotides were annealed by mixing, heating to 100 °C for 3 min, and cooling slowly to room temperature, creating a double-stranded product.

To conduct the EMSA, 6 µg of purified recombinant human heat shock transcription factor (Affinity Bioreagents, Golden CO) or 12 µg of *B. malayi* nuclear extract were pre-incubated for 10 min on ice in a total volume of 40 µl in 10 mM Tris–HCl (pH 8.0), 1mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, 12.5 µg/ml of poly(dI/dC) (Sigma Chemical, St Louis, MO) and 300 µg/ml of bovine serum albumin. The labeled double-stranded oligonucleotides (20,000 cpm) were then added and the mixture incubated for 15 min at room temperature. The samples were loaded onto a native 7% (w/v) polyacrylamide gel (containing 50 mM Tris–borate (pH 8.0), 1 mM EDTA) and subjected to electrophoresis at 35 mA for 2 h. The gel was then dried and subjected to autoradiography. In experiments involving the addition of competitor, the hHSTF was pre-incubated with a 100-fold excess of cold competitor oligonucleotide for 10 min prior to adding the radioactively labeled product.

† http://www.tigr.org/tdb/e2k1/bma1

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