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Analysis of Transcriptional Regulation of Tetracycline Responsive Genes in *Brugia malayi*

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Abstract

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

Keywords

filariasis; transfection; promoter; *Wolbachia*

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1. Introduction

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

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

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

Studies of transcriptional regulation in the human filaria have been hampered by a paucity of tools to manipulate these parasites. However, significant progress has been made employing biolistics to transiently transfect isolated embryos of *B. malayi* [18]. Transfected embryos, although developmentally incompetent, may be maintained in culture for several days [18]. This system has been used to demonstrate that the core domains of many *B. malayi* promoters are exceptional [19–21]. In place of canonical CAAT and TATA box elements located roughly 30nt upstream of the start site of transcription, the core of the *B. malayi* promoters analyzed to date is localized to a pyrimidine rich region located just upstream of the spliced leader addition site [22]. Although the transient transfection system has been used extensively to study the core promoter of *B. malayi*, studies applying this method to investigate transcriptional regulatory regions in *B. malayi* promoters have been much more limited. To date, the only study investigating cis-acting promoter regulatory element has

used a mutant *B. malayi* promoter containing a synthetic ecdysone response element to demonstrate that a functional ecdysone signaling pathway exists in this parasite [23]. No studies have been reported identifying endogenous cis-acting regulatory elements in *B. malayi* promoters.

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

2. Materials and Methods

2.1 Identification of candidate genes

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

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

2.2 Preparation of promoter constructs

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

The series of 30nt linker scanner mutants encompassing the BmHSP70 promoter were prepared as previously described [20]. The 10nt and smaller substitution mutants were

constructed using the Gene Tailor *in vitro* mutagenesis kit (Invitrogen) as previously described [22].

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

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

3. Results

In previous studies, *B. malayi* promoter activity has been assayed using a dual luciferase assay, in which activity from experimental promoters driving the expression of a firefly luciferase reporter gene was normalized by co-transfection with a construct consisting of the BmHSP70 promoter driving the expression of a renilla luciferase reporter [19–22]. However, the BmHSP70-renilla construct could not be used in studies of the effect of tetracycline on promoter activity, as microarray studies had suggested that expression of the *B. malayi* HSP70 homologue was itself up-regulated in response to tetracycline treatment [17]. To identify promoter sequence that might be utilized as a normalization control, the microarray data were examined to identify putatively strong promoters whose expression was unaffected by tetracycline, as described in Materials and Methods. Based upon this analysis, the BmMFSP promoter was chosen as a candidate normalization control. The putative promoter of the BmMFSP gene was cloned into the pRLnull renilla luciferase reporter plasmid and the construct biolistically transfected into isolated *B. malayi* embryos. The transfected embryos were cultured in the presence and absence of tetracycline, and assayed for renilla reporter activity, as described in Materials and Methods. The amount of renilla luciferase activity produced by the embryos cultured in the presence of tetracycline did not differ from that produced by transfected embryos cultured in the absence of the drug (Figure 1). These data confirmed that the activity of the BmMFSP promoter was unaffected by tetracycline.

The construct containing the BmMFSP promoter (BmMFSP (−1194 to −1)/ren) was then used as an internal control for transfection in a dual luciferase assay to test the response of three promoters (BmHSP70, BmRPL13 and BmRPS4) whose mRNA levels were found to

be increased in response to tetracycline treatment in intact adult female parasites *in vivo*. The level of firefly reporter gene expression from all three promoters was found to be significantly increased in transfected embryos treated with tetracycline, when compared to embryos transfected in parallel that were not exposed to tetracycline (Figure 2). These data suggested that the increase in mRNA levels in all three of these transcripts in tetracycline treated parasites was at least partly the result of up-regulation of transcription at the promoter level.

The BmHSP70 promoter has previously been mapped to 2–3nt resolution [20]. The promoter was found to contain four essential domains in the region located from –278 to –32 relative to the start of the ORF (Figure 3) [20]. The largest essential domain extended from –53 to –32, and flanked the SL addition site. Subsequent studies have demonstrated that the region surrounding the SL addition site represents the essential core promoter domain of many *B. malayi* genes [22]. The promoter also contained one active heat shock response element (HSE), located at –235 to –224 relative to the start of the ORF [20]. Because a large amount of information was available concerning the structure of the BmHSP70 promoter, it was chosen as the candidate promoter for mapping studies to identify the cis-acting factors responsible for the tetracycline response.

To roughly map the motif responsible for the tetracycline response, a series of 30nt substitution mutants previously developed as part of the effort to map the essential domains of the BmHSP70 promoter were employed [20]. These linker scanner mutants encompassed the region extending from –336 through –58 relative to the start of the ORF (Figure 3). This region included the entire promoter domain mapped by previous studies [20] with the exception of the core promoter domain surrounding the spliced leader (SL) addition site (Figure 3). The core promoter domain was not included in the analysis of tetracycline responsiveness, as mutation of this region resulted in a complete loss of promoter activity [20]. The 30nt substitution mutants were transfected into embryos, the transfected embryos cultured in the presence or absence of tetracycline and assayed for luciferase activity, as described above. The majority of the clones exhibited a proportional increase in promoter activity that was similar to that seen with the wild type promoter (Figure 4). However, mutation of two 30nt regions resulted in constructs in which the tetracycline response was dramatically reduced. These included regions –276 to –247 and –216 to –187. The –276 to –247 region included a domain that was previously shown to be essential for core promoter activity (Figure 3), and, as expected, mutation of this region reduced the basal level of promoter activity by roughly 92% (Figure 4, Panel A). This suggested that the loss of tetracycline responsiveness in this construct might have been a result of the almost complete loss of promoter activity as a whole in this mutant. In confirmation of this hypothesis, mutation of the –276 to –270 and –263 to –247 regions flanking the –269 to –264 essential domain exhibited a response to tetracycline that was indistinguishable from that of the wild type promoter (Figure 5). Therefore, the diminished response to tetracycline observed in the –276 to –247 mutant was a result of the almost complete elimination of promoter activity resulting from mutation of the essential domain.

In contrast, to the –276 to –247 region, mutation of the –216 to –187 region resulted in a construct whose basal activity in the absence of tetracycline was 187% of that of the basal activity of the wild type promoter (Figure 4, Panel A), confirming previous studies demonstrating that this region was not essential for basal promoter activity [20]. However, tetracycline responsiveness in this mutant was dramatically reduced relative to that seen in the wild type promoter (Figure 4, Panel B), suggesting that this region encoded the motif responsible for the tetracycline response. Analysis of the –216 to –187 region with the transcription factor binding site prediction program TRANSFAC [25] predicted that this region contained a putative TATAA box, located at positions –209 to –205 (Figure 3). The

TATAA box makes up an essential part of many eukaryotic promoters and interacts with several proteins that make up the RNA polymerase pre-initiation complex, including the TATAA box binding protein (TBP) and transcription factor TFIID [26]. Previous studies that mapped the essential domains of the BmHSP70 promoter [19, 20] demonstrated that the putative TATAA box was neither necessary nor sufficient for BmHSP70 basal promoter activity. However, it was possible that the putative TATAA box was involved in the up-regulation of transcription in response to tetracycline treatment. In support of this hypothesis, mutation of residues -216 to -207 (which overlap the putative TATAA box) resulted in a loss of the tetracycline response, while mutation of residues -204 to -197 and -196 to -187 (located downstream of the putative TATAA box) retained tetracycline responsiveness (Figure 5). To confirm the role of the TATAA box in the tetracycline response the TATAA box (residues -209 to -205) was mutated, the mutant construct used to transect *B. malayi* embryos, and the mutant promoter's response to tetracycline assayed as described above. Mutation of the TATAA box eliminated the response to tetracycline, confirming that this motif was essential in mediating the tetracycline response (Figure 5).

4. Discussion

In previous studies, increases in both mRNA and proteins derived from several nuclearly encoded genes of *B. malayi* have been observed in response to treatment with tetracycline [17, 27]. The underlying rationale for these experiments was that the expression of nuclearly encoded proteins involved in the host-endosymbiont relationship would be perturbed by elimination of the endosymbiont. The data presented above confirm that transcription driven from the promoters of some genes whose expression has previously been shown to be affected by tetracycline treatment is up-regulated in response to tetracycline treatment. This finding suggests that at least part of the increased expression of some gene products in response to tetracycline is controlled at the level of transcription.

Previous studies have utilized transient transfection to identify the core promoter domains of *B. malayi* genes [19–22]. In addition, this technique has been used to demonstrate the presence of an ecdysone responsive regulatory pathway in *B. malayi*, employing a synthetic construct consisting of the BmRPS12 promoter modified to contain a synthetic ecdysone response element [23]. The current report demonstrating up-regulation of the BmRPL13, BmRPS4 and BmHSP70 promoters in response to tetracycline represents the first direct demonstration of transcriptional regulation by a small molecule in a native *B. malayi* promoter.

Tetracycline treatment, which results in the elimination of the *Wolbachia* endosymbiont, would be expected to be stressful to the host. This would in turn be expected to result in the up-regulation of transcription of proteins involved in the generalized stress response, including BmHSP70. In the heat shock proteins, up-regulation in response to stress is mediated by heat shock transcription factors, which act by binding to heat shock elements (HSEs) in the promoter, up-regulating transcription [28]. The BmHSP70 promoter contains a functional HSE [20], and if tetracycline treatment was causing up-regulation of transcription through the stress pathway, one would expect that mutation of the HSE in the BmHSP70 promoter would have eliminated the tetracycline response. However, mutation of the HSE did not result in any change in the response of the BmHSP70 promoter to tetracycline (c.f. construct -246 to -217, Figure 4). This suggests that the up-regulation of transcription by tetracycline involves a regulatory pathway that is distinct from the generalized stress response pathway.

Previous studies have identified a TATAA box-like sequence present in the BmHSP70 promoter [19, 29]. Initially, it was believed that this motif would represent part of the core of

the BmHSP70 promoter. However, mutation of this motif has little effect on basal promoter activity (Figure 4, Panel A, construct -216 to -187 and [20]), demonstrating that the motif is not part of the core promoter. However, the mapping studies presented above demonstrate that the TATAA box-like motif is necessary for tetracycline-mediated up-regulation of the BmHSP70 promoter. There are two possible explanations for this finding. First, it is possible that the TATAA-box-like element actually represents an enhancer-like sequence that binds a particular transcription factor, up-regulating transcription from the core promoter domain surrounding the SL addition site in the BmHSP70 promoter. Alternatively, a global analysis of mammalian promoter structure has brought into question the paradigm that most eucaryotic promoters utilize TATAA and CAAT boxes as their core promoter domains [30]. Such prototypical core promoter domains are now known to be lacking in many mammalian promoters. Promoters lacking such prototypical core domains are generally found in constitutively expressed mammalian genes. In these genes, transcription start sites are diffused over a relatively wide area, and the promoters of such genes are characterized by CpG islands [31]. TATAA-box containing promoters are generally found in mammalian genes whose transcription is regulated, and transcription start sites in such genes are confined to a single nucleotide or a small number of nucleotides in a confined area [31]. Previous studies have demonstrated that the transcription start sites of the BmHSP70 gene are diffuse in nature [19]. It is thus logical to hypothesize that transcription of the BmHSP70 gene might be regulated by the interaction of two distinct types of core promoter domains. The first of these would be diffuse, and would occur during basal transcription of the BmHSP70 gene, or in response to generalized stress. The second domain would involve transcription induced by tetracycline and would involve a specific transcriptional start mediated by the TATAA box. This latter hypothesis could be tested by analyzing the transcriptional start points of transgenic mRNAs produced from the BmHSP70 promoter in the presence of tetracycline.

The experiments described above provide direct evidence that the TATAA box is necessary for up-regulation of promoter activity in the presence of tetracycline, but the experiments do not directly address the question if the TATAA box alone is sufficient to mediate this response. However, tetracycline responsiveness was altered when only two regions of the BmHSP70 promoter were mutated. These included a region formerly shown to be essential for basal promoter activity and the TATAA box. Mutation of the former domain resulted in a complete loss of promoter activity; since the promoter in this construct was completely inactivated, it is likely that the lack of tetracycline responsiveness seen with this mutant was a result of the overall inactivation of the promoter. Thus, the TATAA box was found to be the only region which when mutated resulted in a construct that retained basal promoter activity but which lost its ability to respond to tetracycline. This finding provides indirect evidence suggesting that the TATAA box is both necessary and sufficient to mediate the tetracycline response. Direct confirmation of this hypothesis will require introducing the TATAA box into a promoter that is not responsive to tetracycline and demonstrating that transcription from this mutant is now up-regulated in response to tetracycline.

Tetracycline treatment of *B. malayi* results in the clearance of the wBm endosymbiont and concurrent changes in the mRNA pools of several nuclearly encoded genes [17]. The data presented above suggest that at least some of this change is affected at least in part through changes in transcription from the affected genes. It is tempting to speculate that the changes in gene expression associated with tetracycline treatment are a result of the parasite's response to the loss of its endosymbiont. However, the data presented above do not rule out the possibility that tetracycline is having an effect on these genes through a mechanism that is independent of its effect on the wBm endosymbiont. More work dissecting how tetracycline effects transcription of these genes will be needed to differentiate these possibilities.

Tetracycline has been used in conjunction with the Tn10 tetracycline resistance operon of *Escherichia coli* to develop a very tightly controlled conditional expression system for eucaryotic cells [32]. The data presented above suggest that tetracycline may induce gene expression of some *B. malayi* genes, making it tempting to speculate that a similar system might be developed for *B. malayi* that could be based upon endogenous tetracycline responsive promoters. Such a conditional expression system, when coupled with the stable transfection method recently reported for *B. malayi* [33] might prove to be a powerful approach to studying gene function in this parasite. However, tetracycline generally has no effect on mammalian cells. In contrast, tetracycline treatment of *B. malayi* results in elimination of the *Wolbachia* endosymbiont, resulting in sterilization and eventually death of the parasite. This would complicate the interpretation of any phenotype resulting from tetracycline-mediated induction of transgene expression by such a conditional expression system. Exploring the use of tetracycline analogs [34], which might induce expression of the appropriate genes without affecting the viability of the *Wolbachia* endosymbiont could be a way to overcome this difficulty.

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Abbreviations

BmHSP70	<i>Brugia malayi</i> 70 kDa heat shock protein
BmRPL	<i>Brugia malayi</i> large subunit ribosomal protein
BmRPS	<i>Brugia malayi</i> small subunit ribosomal protein
HSE	Heat shock element
MFSP	major facilitator superfamily protein
nt	nucleotides
ORF	Open reading frame
PCR	polymerase chain reaction
SL	Spliced leader
TBP	TATAA box binding protein
wBm	<i>Wolbachia</i> endosymbiont of <i>B. malayi</i>

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Figure 1. Reporter gene activity in embryos transiently transfected with BmMFSP (-1194 to -1)/ren in the presence or absence of tetracycline

Embryos were transiently transfected as described in Materials and Methods, cultured for 48 hours in the presence or absence of $40 \mu\text{g ml}^{-1}$ tetracycline and assayed for renilla luciferase reporter gene activity. Data are expressed as relative light units (RLU) mg^{-1} parasite protein.

Figure 2. Reporter gene activity in embryos transfected with candidate tetracycline responsive promoters in the presence or absence of tetracycline

Embryos were transfected with promoter constructs, cultured in the presence or absence of tetracycline and assayed for reporter gene activity as described in Materials and Methods. Columns indicate the mean, and error bars the standard deviations, of reporter gene activity in six independent transfections. Asterisks indicate promoters whose activity was significantly up-regulated in the presence of tetracycline ($p < 0.01$).

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      -331      -321      -311      -301      -291      -281      -271      -261      -251
CCTAGC CCCAATCACT TATTATCTTT GATATTCTGC TAGCAGCACT CGCACACTGA CTATTGGAGT ACACTCTCTG CCTGTGCACT

      -241      -231      -221      -211      -201      -191      -181      -171      -161
GAACATTCTG TAGCTTTCCA GAACATTCTGA TGTGTTCCAC ATATAAAGGA ACTGAGCGAT GGCTGCCTCG CTAGTGGTGA GTCGTCGTTG

      -151      -141      -131      -121      -111      -101      -91      -81      -71
TACAGTGCTC GGTTGCGTTG TCGGTTGTTT GGGCTAGTGG AGGGGGGGGG GGGGGAAGTA GTATGCGTGG ATTGTGTGTT TGACGCTGCT

      -61      -51      -41      -31
TTATACGTGT GGTCTAGTTT TAGGCGTGCT CGTTGTTGAT

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Figure 3. Structure of the BmHSP70 promoter

Areas highlighted in grey indicate sequences which, when mutated, resulted in the loss of 50–90% of basal promoter activity, while sequences highlighted in black represent those which, when mutated, resulted in the loss of >90% of basal promoter activity [20]. The functional HSE is indicated by a dotted box, and the TATAA-box like element by underlining.

Figure 4. Reporter gene activity in embryos transfected with 30nt substitution mutants of the BmHSP70 promoter in the presence or absence of tetracycline

Embryos were transfected with promoter constructs, cultured in the presence or absence of tetracycline and assayed for reporter gene activity as described in Materials and Methods. Panel A: Reporter gene activity of promoter constructs in the presence or absence of tetracycline. Columns indicate the mean and error bars the standard deviations of reporter gene activity in six independent transfections. Panel B: Extent of up-regulation of reporter activity in the presence of tetracycline. Asterisks indicate constructs whose activity was significantly up-regulated in the presence of tetracycline ($p < 0.01$).

Figure 5. Reporter gene activity in embryos transfected with small substitution mutants of the BmHSP70 promoter in the presence or absence of tetracycline

Embryos were transfected with promoter constructs, cultured in the presence or absence of tetracycline and assayed for reporter gene activity as described in Materials and Methods. Panel A: Reporter gene activity of promoter constructs in the presence or absence of tetracycline. Columns indicate the mean and error bars the standard deviations of reporter gene activity in six independent transfections. Panel B: Extent of up-regulation of reporter activity in the presence of tetracycline. Asterisks indicate constructs whose activity was significantly up-regulated in the presence of tetracycline ($p < 0.01$).