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Robust heat-inducible gene expression by two endogenous *hsp70*-derived promoters in transgenic *Aedes aegypti*

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Abstract

Aedes aegypti is an important vector of the viruses that cause dengue fever, dengue hemorrhagic fever, and yellow fever. Reverse genetic approaches to the study of gene function in this mosquito have been limited by the lack of a robust inducible promoter to allow precise temporal control over a protein-encoding or hairpin RNA transgene. Likewise, investigations into the molecular and biochemical basis of vector competence would benefit from the ability to activate an anti-pathogen molecule at specific times during infection. We have characterized the ability of genomic sequences derived from two *Ae. aegypti hsp70* genes to drive heat-inducible expression of a reporter in both transient and germline transformation contexts. *AaHsp70*-luciferase transcripts accumulated specifically after heat shock, and displayed a pattern of rapid induction and decay similar to endogenous *AaHsp70* genes. Luciferase expression in transgenic *Ae. aegypti* increased by ~25-50 fold in whole adults by four hours after heat-shock, with significant activity (~20 fold) remaining at 24 hr. Heat-induced expression was even more dramatic in midgut tissues, with one strain showing a ~2500-fold increase in luciferase activity. The *AaHsp70* promoters described could be valuable for gene function studies as well as for the precise timing of the expression of anti-pathogen molecules.

Keywords

HSP70; promoter; *Aedes aegypti*; transgenic; mosquito; heat shock; stress

Introduction

Aedes aegypti is a significant vector of disease agents, and is primarily responsible for transmission of the viruses that cause yellow fever, dengue fever, and dengue hemorrhagic fever (Tatem et al. 2006). Current efforts to control *Ae. aegypti* populations such as insecticides and habitat removal have not yet been sufficient to interrupt transmission of these disease agents. Genetic control strategies are being developed as an additional alternative to aid in reducing disease transmission, with a number of recent reports describing the development of novel female-killing or pathogen-resistant transgenic mosquito strains (Fu et al. 2010; Isaacs et al. 2011; Kokoza et al. 2010; Meredith et al. 2011; Wise de Valdez et al. 2011).

The generation of pathogen-resistant, genetically sterile or female-killing transgenic mosquitoes depends upon the ability to drive the expression of one or more foreign genes in an appropriate temporal and spatial manner. Likewise, basic research studies addressing

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questions of gene function using ectopic expression or gene knockdown must often be precisely timed in order to avoid developmentally disruptive phenotypes. A wide range of promoter elements have been characterized and utilized to drive transgene expression or gene knockdown in *Aedes aegypti*, including the salivary gland specific *apyrase* (Coates et al. 1999) and *30K* (Mathur et al. 2010) promoters, the midgut-specific *carboxypeptidase* (*CP*) promoter (Moreira et al. 2000), the fat-body specific *vitellogenin* (*Vg*) promoter (Kokoza et al. 2001), ovary-specific vitellogenin receptor and *nanos* promoters (Adelman et al. 2007; Cho et al. 2006), the testes-specific $\beta 2$ *tubulin* promoter (Smith et al. 2007) and the full body *polyubiquitin* (*PUB*) promoter (Anderson et al. 2010). While these promoter elements provide a range of spatial controls, the only temporal controls (*Vg*, *CP*) are linked to bloodfeeding. Uncoupling transgene expression from the acquisition of a bloodmeal would substantially broaden the types of experiments which could be performed in this species. For example, expressing a foreign gene or establishing a gene knockdown phenotype early after adult emergence would allow investigations into host seeking behavior. Similarly, restricting expression until long after a bloodmeal is taken would allow investigations into host-pathogen interactions, where gene products could be expressed at a specific point in a pathogens' life cycle.

In *Drosophila*, such precise temporal control is achieved using the *hsp70* promoter, where both ectopic expression and gene knockdown experiments are common (Aigaki et al. 1991; Lam and Thummel 2000; Read et al. 1992). However, significant basal expression in *Aedes aegypti* has limited the utility of this promoter in *Ae. aegypti* (Morris et al. 1991). Ultimately, no rigorous analysis of any heat shock promoter has been reported for this mosquito.

Recently we characterized the gene structure and expression patterns of six members of the *Hsp70* family in *Aedes aegypti* (Gross et al. 2009). In an effort to identify an *Ae. aegypti* promoter capable of providing strict temporal control over a transgene, we placed the firefly luciferase ORF under the control of sequences derived from the genomic region upstream of the *AaHsp70Aa* or *AaHsp70Bb* genes. The ability of these *AaHsp70* sequences to drive luciferase expression was examined in transient cell and embryo assays, as well as in the context of *Ae. aegypti* chromosomal insertions via the transposon *Mos1*. Both promoter constructs were found to drive heat-inducible expression throughout adult female mosquitoes.

Results

Bioinformatic analysis of potential heat shock factor binding sites

Hsp70 genes require specific enhancers in order to respond to heat stress, termed heat shock elements (HSE) (reviewed in (Sakurai and Enoki 2010)). The consensus HSE consists of the basic pattern nGAAn (where n is any nucleotide) arranged in a cluster of three or more head-tail repeats. We analyzed the genomic intervals between three *AaHsp70* inverted gene pairs (Gross et al. 2009) for the presence and abundance of HSEs. Only a single sequence corresponding to a canonical nTTCn-nGAAn-nTTCn trimeric HSE was identified, and was located about midway between genes *AaHsp70Aa* and *AaHsp70Ab*. The heat shock transcription factor (HSF) binds to HSE as a trimer, and is known to allow one of its three subunits to wobble (reviewed in (Sakurai and Enoki 2010)). Therefore, we also searched for potential trimeric HSE sites where one of the three nGAAn units was allowed to be randomized (Table 1). While tail-tail and head-head arrangements of the nGAAn units have been shown to bind HSF equally well, we observed a preference for the head-head arrangement in *Ae. aegypti Hsp70* intergenic sequences (Table 1). However, the most common HSE-like element was the step/gap type, which was significantly enriched in all three intergenic regions (Table 1). This suggests that in *Ae. aegypti*, the middle subunit may

contain the greatest tolerance for deviation from consensus. Alignment of all 28 step/gap type HSE sequences revealed a preference for the nucleotides GA separating the first and second units, with a strong preference for an A/T in the first position of the third unit (Fig. 1). This consensus may help to identify other *Ae. aegypti* genes that are transcriptionally activated under heat stress.

Heat-induced gene expression has been shown to be proportional to the number of consecutive nGAAn subunits present upstream of the transcriptional start site (Fernandes et al. 1995). To determine the size and location of any HSE clusters upstream of *AaHsp70* genes, the genomic positions of HSEs identified in Table 1 were compared. With the exception of *AaHsp70Cb*, all genes contained a pattern immediately upstream (-50 to -90 nt for *AaHsp70Aa*) of the start of transcription consisting of 4 consecutive subunits, a 6 bp spacer, and an additional 3 subunits. Functionally, this may be similar to an 8-subunit cluster. Both *AaHsp70Aa* (-672 to -702 nt) and *AaHsp70Bb* (-936 to -966 nt) contained a second cluster of 6 subunits, while *AaHsp70Aa* alone contained a third large cluster of 5 subunits (-1264 to -1284). The relative positions of HSE clusters in the *AaHsp70Aa-Ab* and *AaHsp70Ba-Bb* intergenic regions are shown in Fig. 2.

AaHsp70 promoter testing in cells and embryo assays

Upstream sequences derived from all six *AaHsp70* genes (Gross et al. 2009) were tested for core promoter activity in cultured mosquito cells; with regions derived from *AaHsp70Aa* and *AaHsp70Bb* as the only ones able to drive the robust expression of a reporter (not shown). In order to test whether all the elements required for steady-state repression and heat activation were present upstream of these genes, genomic segments derived from *AaHsp70Aa* or *AaHsp70Bb* containing variable numbers of HSE clusters were placed upstream of the reporter gene, firefly luciferase (FFluc) (Fig. 2). *AaHsp70Aa*-FFluc and *AaHsp70Bb*-FFluc constructs were transfected into *Ae. aegypti* Aag2 cells or injected directly into pre-blastoderm *Aedes aegypti* embryos. A second plasmid which controlled the expression of Renilla luciferase (Rluc) in a heat shock-independent manner served as an internal normalization control. Following heat shock, the ratio of FFluc to Rluc was determined (Fig. 2). Luciferase activity driven from all *AaHsp70* constructs was found to be significantly upregulated in both cells and embryos in response to heat shock. For *AaHsp70Aa* constructs, the level of luciferase activity following heat shock was similar between all three test constructs, indicating that all of the information needed to respond to heat shock was present between -1373 and +182 relative to the start of transcription, which included the main 8-member cluster as well as the 6- and 5-member clusters (Fig. 2A). While all *AaHsp70Bb*-based constructs demonstrated significant differences between control and heat shock expression, significantly less heat-induced expression was observed for the *AaHsp70Bb*-787 construct as compared with *AaHsp70Bb*-1456, *AaHsp70Bb*-1696, and *AaHsp70Bb*-2858. This indicates that for this promoter, critical information (potentially the 6-member HSE cluster) for heat shock-based transcriptional activation is located in the region between -787 and -1456. In both cases the 8-member HSE cluster for the opposite gene (*AaHsp70Ab* and *AaHsp70Ba*) was expendable, suggesting little cross-talk between these promoters. Based on these data, promoters *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456 were chosen as the best candidates for further experimentation in transformed *Ae. aegypti*.

Generation of transgenic *Ae. aegypti*

In order to determine the ability of *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456 promoter fragments to provide for the strict temporal control of a transgene in the context of the mosquito chromosome, *Mos1* transformation constructs were designed (Fig. S1, A & C) to include each promoter-reporter cassette, and were introduced into *Ae. aegypti* embryos. G₁ progeny obtained from surviving individuals were screened as larvae for DsRed expression

under the control of the *PUB* promoter (Anderson et al. 2010), with putative transgenic individuals identified in three *AaHsp70Aa*-1373 pools and four *AaHsp70Bb*-1456 pools (Table S1). Genomic integrations were confirmed using Southern analysis (Fig. S1, B & D), and all transgene cassettes were stably inherited to the G₂ generation (Table S1).

Validation of *AaHsp70* promoters in adult female *Ae. aegypti*

In order to determine if the *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456 promoters contained all the information required to respond to a heat shock with a burst of transcription in the context of the mosquito chromosome, we measured the total firefly luciferase activity in adult transformed individuals from all seven transgenic strains. Consistent with the data from cell and embryo assays, a significant increase in FFluc activity was observed in all transgenic strains as early as 1 hr post heat shock for both promoters (Fig. 3). FFluc activity was consistently about 10-13 times higher in heat shocked individuals than the corresponding untreated siblings at this time point. A clear difference in the absolute expression levels of the two promoters was also observed, with the *AaHsp70Aa*-1373 fragment driving the expression of ~10 times more FFluc before and after heat shock (Fig. 3). Statistical analysis of FFluc levels among all seven control groups using Tukey's HSD test confirmed that the basal expression levels of the *AaHsp70Aa*-1373 lines was uniformly higher than those of *AaHsp70Bb*-1456 lines. A similar comparison between all seven sets of heat-shock values revealed a similar, but less dramatic trend, with $Aa\#P10 > Aa\#P5 > Aa\#P2 = Bb\#P1 = Bb\#P6 > Bb\#P7 > Bb\#P1$. These results confirm that both the *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456 promoters are capable of driving the heat-induced expression of a foreign gene in transgenic *Ae. aegypti*, albeit at different overall levels, potentially due to the extra 5-member HSE cluster present in the *AaHsp70Aa*-1373 fragment.

Endogenous *hsp70* transcripts accumulate rapidly following heat shock, and are subject to degradation following the successful refolding/recycling of damaged proteins (Petersen and Lindquist 1989). In order to determine if FFluc transcripts under the control of *AaHsp70* fragments mirrored this pattern, transcript abundance was measured by Northern analysis at various times following heat shock. Analysis of RNA derived from heat-shocked *AaHsp70Aa*-1373 line #P10 individuals revealed a pattern of luciferase mRNA levels similar to native *AaHsp70* genes described previously (Gross et al. 2009), with abundant transcripts immediately following heat shock and a subsequent strong decrease by 24 hours (Fig. 4A). Phosphorimager-based quantitation revealed that FFluc transcripts were 20, 18, 13, and 6 percent of maximal *AaHsp70* expression at 0, 1, 2, and 4 hours, respectively. Considering that the probe used to detect *AaHsp70* transcripts was capable of hybridizing to all 12 *AaHsp70* gene transcripts, while the luciferase probe would recognize only those transcripts resulting from *AaHsp70Aa*-1373-FFluc expression, this indicates that the *AaHsp70Aa*-1373 promoter is capable of driving transcription at a rate comparable to its endogenous counterpart.

Similarly, we measured the accumulation of FFluc over time in *AaHsp70Aa*-1373 line #P10 and in *AaHsp70Bb*-1456 line #P6. Luciferase assays confirmed a pattern of protein abundance similar to that of transcript abundance, though there was a delay in the time needed to reach peak levels (Fig. 4, B & C). FFluc levels were highest at 4 hours following heat shock for both promoters, reaching levels 25-50 times that of untreated females. Substantial luciferase activity remained (16-20 fold above control levels) at 24 hrs post heat shock even though mRNA transcripts could no longer be detected, indicating that FFluc is fairly stable under the conditions used herein. Luciferase activity in *AaHsp70Bb*-1456-FFluc transformed females followed a similar pattern as *AaHsp70Aa*-1373, but again with lower overall expression levels (Fig. 4C).

Heat induced expression of native Hsp70 has been previously confirmed in *D. melangaster* in all tissues assayed, including brain, salivary glands, midgut and female gonadal disks (Krebs and Feder 1997). Similarly, we have shown that *AaHsp70* gene transcription is activated in similar tissues following heat shock in *Ae. aegypti* (Gross et al. 2009). In order to determine the pattern of *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456-driven expression in specific tissues, we performed luciferase assays on dissected heads, midguts (sugar-fed), salivary glands and ovaries (sugar-fed) in each transformed line (Fig. 5). Head tissue is enriched for neurons, a common site for arbovirus amplification (Linthicum et al. 1996), and also contains significant amounts of fat body, the main immune organ of the mosquito. All three transgenic lines derived from the *AaHsp70Aa*-1373-FFluc construct displayed a ~10 fold increase in luciferase activity in heads at 2-4 hrs following heat shock (Fig. 5A). Heat shock-induced expression was much more dramatic in *AaHsp70Bb*-1456-derived lines, due to significantly lower basal levels of expression in this tissue, as determined by Tukey's HSD test (Fig. 5A). In midguts, heat-induced luciferase levels were significantly increased in all lines, though there was much more variation between transgenic strains as compared to heads. No clear differences between the two promoters was observed, with the *AaHsp70Bb*-1456 promoter yielding transgenic strains with both the least and the greatest level of induction as measured by fold change. In particular, *AaHsp70Bb*-1456 line #P3 showed the greatest change between control and heat shock, with ~2600-fold induction of FFluc. Differences in fold change were again largely due to differences in basal levels of expression, which spanned three orders of magnitude (Fig. 5B). Heat-induced activation of FFluc was also observed in salivary glands (Fig. 5C) and ovaries (Fig. 5D). These data confirm that *AaHsp70* promoters are capable of driving the temporally-controlled expression of a transgene in a wide variety of mosquito tissues.

Discussion

Overall, we found that genomic sequences derived from regions upstream of two *AaHsp70* genes are capable of driving the heat-inducible expression of a marker gene both transiently in *Aedes aegypti* cells and embryos and through the stable integration of a transgene. Both the *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456 promoters were found to be capable of transcriptional activation in response to a 1 hr treatment at 39°C in whole adults as well as individual tissues.

We found that the intergenic regions separating all three pairs of *AaHsp70* genes we examined were highly enriched for nGAAn-nTTCn and nTTCn-nnnnn-nTTCn HSE motifs, with the latter forming a consensus nTTCg-aGNRn-wTTCn. In flies, multiple trimeric HSEs arranged in clusters show much greater cooperative binding of HSF than the same number of sites spread at a distance; this increase in HSF binding was associated with increased gene activation at lower temperatures (Fernandes et al. 1995). Our data agree with this, as the *AaHsp70Aa* gene promoter had both the greatest number of large HSE clusters and the strongest basal expression levels. Though FFluc expression was correlated with a higher number of HSE clusters, the large HSE clusters located at the start of transcription of the distal *Hsp70* gene were expendable. It is possible that the distance (>2.5 kb) of these clusters from the transcriptional initiation site was too great to exert a significant influence on transcriptional activation (Fernandes et al. 1995). Ultimately, further investigations will be necessary to clarify the precise role of individual HSE clusters in the heat-induced activation of *AaHsp70* genes.

The appropriateness of which of these two promoters to use in a given experiment will likely depend on the trait that is more important to the investigator: the ability to express as much gene product as possible, or the ability to strongly suppress transcription prior to heat activation. We emphasize, however, that even though the *AaHsp70Aa*-1373 promoter

exhibited basal expression levels $\sim 10\times$ higher than the *AaHsp70Bb*-1456 promoter, we were still unable to detect *AaHsp70Aa*-FFluc transcripts in the absence of heat shock. Thus, even this “higher” level of expression should be considered extremely low compared with most transcripts. It is likely that the sensitivity of the firefly luciferase reporter, combined with its relatively long half-life, played an important role in our ability to detect expression in the absence of heat shock. While the half-life of firefly luciferase was reported to be ~ 3 hours at 37°C (Thompson et al. 1991), our data indicate that this is substantially lengthened at 28°C to between 8-16 hours (Fig. 4). This issue might be avoided with experiments where no protein is produced, such as the expression of hairpin RNAs for gene knockdown, or where protein products can be intentionally destabilized.

In contrast to our inability to detect FFluc transcripts in the absence of heat shock, these transcripts became very abundant immediately after the one hour treatment at 39°C , mirroring the pattern exhibited by the endogenous *AaHsp70* genes (Gross et al. 2009). Surprisingly, luciferase transcripts decreased much more quickly than the corresponding *AaHsp70* transcripts. This is notable because our transformation constructs did not include the native *AaHsp70* 3' untranslated region (UTR). In flies, the 3'UTR is important for heat shock transcript regulation, as it targets messages for de-adenylation and degradation once stress conditions have passed (Dellavalle et al. 1994; Feder et al. 1992; Petersen and Lindquist 1989). Utilizing the 3'UTR sequence from SV40 in place of the native *AaHsp70* 3'UTRs in our transgenic constructs, the stability of luciferase transcripts was expected to increase. These results may indicate that the stability of *AaHsp70* transcripts is regulated differently than in flies, with additional signals present in *AsHsp70* transcripts which identify them as targets for deadenylation during recovery from heat stress.

Luciferase expression levels between the three *AaHsp70Aa*-1373 transgenic lines was fairly consistent in whole adults, heads, ovaries and salivary glands, indicating that the chromosomal position of each *Mos1* insertion did not exert a strong influence on the expression or activation of this promoter. Likewise, with the exception of line #P3, luciferase expression in *AaHsp70Bb*-1456 transgenic strains was similar in whole adults and heads alone. In contrast, for both promoter constructs basal expression levels in the midguts were much more variable; indicating that expression in this tissue is much more susceptible to chromosomal position effects. We note that both transformation constructs included an *attP* recombination site, allowing the re-use of any of these integration sites using PhiC31-mediated recombination (Nimmo et al. 2006).

In conclusion, we have shown that two independent *AaHsp70* promoter regions can activate robust transcription in response to heat shock in *Ae. aegypti*. Such precise temporal control of a transgene will be vital for gene knockdown or ectopic expression studies of genes whose loss/gain would adversely affect the successful progression through development. Uncoupling temporal control of transgene expression from a bloodmeal will allow investigations into events which occur before a mosquito obtains her first meal, such as host-seeking or mating, as well as events which happen late in bloodmeal digestion or oogenesis. Additionally, the precisely timed expression of a transgene can be used to dissect host-pathogen interactions, by allowing the expression of a foreign gene at a specific point in a pathogen's life cycle. Finally, the transgenic strains we have developed here can be used directly as sensors to detect activation of the stress response [as reviewed in (Mukhopadhyay et al. 2003)]. While the stress response of *D. melanogaster* has been studied extensively (as reviewed in Craig 1985; Lindquist 1986), there is only limited understanding of conditions such as environmental contaminants, insecticides, infectious agents or biological processes which may induce the stress response in *Aedes aegypti* (Benoit et al. 2011; Muturi et al. 2011).

Experimental Procedures

Cell culture and transient embryo assays

Aedes aegypti Aag2 cells were maintained at 28°C and 5% CO₂ in Schneider's Drosophila medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Twelve-well plates were seeded with to be transfected with luciferase promoter plasmid constructs. A total of 0.5 µg of DNA was transfected with 12.5 µl of Effectene into Aag2 cells at approximately 80% confluency with a 1:1 ratio of experimental plasmid and the pRL Renilla luciferase control plasmid (Promega). *Aedes aegypti* embryos were collected from bloodfed females and injected 30 to 90 minutes after being laid with 300 ng/µl experimental plasmid and 15 ng/µl pRL in 1X injection buffer (Jasinskiene et al. 1998). Twenty-four hours following transfection/injection, cells/embryos were heat shocked at 37°C/39°C, respectively, for one hour and were allowed to recover for 1 hr at 28°C prior to harvest. Dual luciferase reporter assays (Promega) were performed on cell lysates after active cell lysis using rubber policemen according to the manufacturer's protocol for 12-well plates. Embryos were crushed in a total of 100 µl of passive lysis buffer (Promega) and luciferase assays were performed as for cell lysates. Luciferase activity was measured using a Hidex Chameleon plate reader (Turku, Finland).

Mosquito assays

The Liverpool strain of *Aedes aegypti* was reared at 28°C and approximately 60% relative humidity with a 14:10 daylight cycle. Larvae were fed pulverized fish food and reared ~ 400 per pan in 4 liters of reverse osmosis purified water until pupation. Adult mosquitoes were maintained on sucrose and were bloodfed using artificial membrane feeders and defibrinated sheep blood (Colorado Serum Company, Denver, CO). Mosquitoes were heat shocked at 3-6 days post eclosion for one hour at 39°C as described previously (Gross et al. 2009). Adult females were frozen individually for luciferase assays, or in groups of 6 for RNA extraction. Tissues were dissected 1-4 hours post heat shock and snap frozen in pools of 10. Adults (100 µl) and tissues (50 µl) were homogenized in 1X passive lysis buffer and luciferase assays were performed according to the manufacturer's protocol (Promega, Dual Luciferase Assay, firefly reading only).

Construction of AaHsp70 promoter-reporter plasmids

Sequences corresponding to genomic position 380841-383453 and position 404011-406759 on supercontig 1.680 were amplified from *Aedes aegypti* Liverpool genomic DNA using Platinum *Pfx* polymerase chain reaction (PCR; Invitrogen, Carlsbad, CA) (2× *Pfx* amplification buffer, 0.3 mM dNTPs, 1 mM MgSO₄, 0.3 µM primers, 1 U Platinum *Pfx* DNA polymerase). Prior to amplification, mosquito genomic DNA was digested with either *Sac*II or *Bgl*III to separate inverted *hsp70* gene pairs. Several amplification products were generated and assembled to reconstitute the entire intergenic region between the *AaHsp70Aa*-*AaHsp70Ab* and *AaHsp70Ba*-*AaHsp70Bb* gene pairs. Each genomic interval was cloned into pGL3-Basic upstream of the firefly luciferase ORF, yielding plasmids *AaHsp70Aa*-2447-FFluc and *AaHsp70Bb*-2858-FFluc.

To produce *AaHsp70Aa*-1734-FFluc, *AaHsp70Aa*-2447-FFluc was digested with *Nco*I and *Hind*III, and the resulting 1916 base pair fragment was ligated into the *Nco*I and *Hind*III of pGL3Basic. To produce *AaHsp70Aa*-1383-FFluc, *AaHsp70Aa*-2447-FFluc was linearized with *Mlu*I, and partially digested with *Ase*I to remove 1064bp of sequence. The remaining *AaHsp70Aa*-FFluc/pGL3 backbone was treated with Klenow to produce blunt ends, followed by self ligation to restore circularity. To produce both *AaHsp70Bb*-1696-FFluc and *AaHsp70Bb*-767-FFluc, *AaHsp70Bb* was linearized with *Mlu*I and partial digests were performed using *Eco*RI. The two shortened *AaHsp70Bb*-FFluc/pGL3-Basic containing

fragments resulting from these digests were treated with Klenow and self-ligated. *AaHsp70Bb*-1456-FFluc was obtained by digesting *AaHsp70Bb*-1696-FFluc with *PvuI*, followed by self-ligation of the remaining plasmid sequence. Final *AaHsp70Aa*-1373 and *AaHsp70Bb*-1456 promoters sequences have been deposited to GenBank under accession numbers JN393894 and JN393895, respectively.

To build *Mos1* donor constructs, the *AaHsp70Aa*-1373-FFluc gene cassette was excised from pGL3-Basic using *MluI* and *BamHI* and ligated into the *AscI/BglII* sites of pMos-*PUBDsRed*-5HE-MCS-5HE (Anderson et al. 2010). Similarly, the *AaHsp70Bb*-1456-FFluc gene cassette was excised from pGL3-Basic with *PvuI/BamHI* and was ligated into the *PacI* and *BglII* sites of pMos-*PUBDsRed*-5HE-MCS-5HE.

Mos1-mediated transformation

Transgenic *Aedes aegypti* were produced by injecting Liverpool strain embryos with 0.5 µg/µl donor plasmid and 0.3 µg/µl helper plasmid (pGL3-*PUB-Mos1*) in 1X injection buffer [see above]. All microinjections were performed using a Leica micromanipulator and a FemtoJet microinjector (Eppendorf, Westbury, NY). Needles were produced using a Sutter Instruments (Novato, CA) Model P-2000 needle puller (Heat = 270, Fil = 3, Vel = 37, Del = 250, Pul = 140). Surviving G₀ females were pooled into groups of 20-25 individuals and mated to Liverpool males. G₀ males were mated individually to 5 Liverpool females and then pooled into groups of 24 males and 120 females, as previously described (Anderson et al. 2010). G₁ progeny were screened for DsRed⁺ using a Leica MZ-16FL microscope.

Northern/Southern analysis

Total RNA was extracted from pools of 6 female mosquitoes using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA (5 µg) from each experimental group was electrophoresed in a 1.2 percent agarose, 1X MOPS, 2% formaldehyde gel and blotted onto a positively charged nylon membrane (Immobilon-NY⁺, Millipore, Concord, MA). Genomic DNA was extracted from pools of six females as described previously (Anderson et al. 2010) and was digested with the indicated restriction endonuclease, followed by gel electrophoresis and capillary transfer to a nylon membrane.

For Northern analysis, a probe specific to the luciferase open reading frame was generated by digesting pGL3Basic (Promega) with *XbaI* and *NcoI*, resulting in an approximately 1.6 kb fragment (Moreira et al. 2000). Probe sequence common to all *AaHsp70* open reading frames was as described previously (Gross et al. 2009). For Southern analysis, probes were produced corresponding to *HindIII* restriction fragments derived from the parent *Mos1* construct, as described previously (Anderson et al. 2010). Random primed probes were labeled with [α -³²P]dATP, using the Amersham Megaprime DNA Labeling System (GE Healthcare, Buckinghamshire, UK). Probes were purified using illustra NICK columns (GE Healthcare) and added to pre-warmed Church's buffer to hybridize overnight at 65°C. Following washes, blots were exposed to Kodak BioMax maximum sensitivity film at -80°C. Hybridization signals were also quantified using a Storm 820 phosphorimager and ImageQuantTL software (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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AaHsp70Aa-Ab1	T	T	T	C	T	A	C	T	G	T	A	T	T	C	A
AaHsp70Aa-Ab2	G	T	T	C	G	A	G	C	G	G	T	T	T	C	T
AaHsp70Aa-Ab3	G	T	T	C	G	A	G	C	A	G	A	T	T	C	G
AaHsp70Aa-Ab4	T	T	T	C	T	A	G	T	T	C	A	T	T	C	A
AaHsp70Aa-Ab5	T	T	T	C	A	C	T	A	G	A	G	T	T	C	C
AaHsp70Aa-Ab6	T	T	T	C	A	G	A	A	A	G	C	T	T	C	A
AaHsp70Aa-Ab7*	G	T	T	C	A	A	G	A	A	T	G	T	T	C	T
AaHsp70Aa-Ab8	G	T	T	C	C	A	G	A	C	C	A	T	T	C	T
AaHsp70Aa-Ab9	T	T	T	C	G	T	C	A	A	T	T	T	T	C	C
AaHsp70Aa-Ab10	A	T	T	C	G	C	T	A	A	C	A	T	T	C	T
AaHsp70Aa-Ab11	A	T	T	C	G	A	G	G	A	C	A	T	T	C	G
AaHsp70Aa-Ab12	T	T	T	C	G	A	A	C	T	A	T	T	T	C	A
AaHsp70Aa-Ab13	G	T	T	C	G	A	G	T	A	C	T	T	T	C	G
AaHsp70Aa-Ab14	G	T	T	C	G	A	G	T	T	C	T	T	T	C	C
AaHsp70Aa-Ab15	C	T	T	C	G	A	T	T	T	G	T	T	T	C	A
AaHsp70BA-Bb1	G	T	T	C	G	A	G	C	G	C	T	T	T	C	C
AaHsp70BA-Bb2	G	T	T	C	G	A	G	C	A	G	T	T	T	C	G
AaHsp70BA-Bb3	A	T	T	C	T	C	G	C	C	C	T	T	T	C	T
AaHsp70BA-Bb4	C	T	T	C	G	A	A	G	T	G	A	T	T	C	A
AaHsp70BA-Bb5	A	T	T	C	G	G	C	C	G	C	A	T	T	C	T
AaHsp70BA-Bb6	C	T	T	C	T	T	G	A	T	G	T	T	T	C	T
AaHsp70BA-Bb7	G	T	T	C	G	A	G	T	A	G	T	T	T	C	G
AaHsp70BA-Bb8	A	T	T	C	G	A	G	T	G	C	T	T	T	C	C
AaHsp70CA-Cb1	T	T	T	C	C	A	C	T	G	T	A	T	T	C	A
AaHsp70CA-Cb2	G	T	T	C	G	A	G	C	A	G	T	T	T	C	C
AaHsp70CA-Cb3	G	T	T	C	G	A	G	T	A	G	T	T	T	C	G
AaHsp70CA-Cb4	T	T	T	C	C	A	T	G	G	A	C	T	T	C	C
AaHsp70CA-Cb5	T	T	T	C	C	G	A	A	A	T	T	T	T	C	A
consensus	N	T	T	C	G	A	G	N	R	N	W	T	T	C	N

Figure 1. Alignment of step/gap type HSEs found in *AaHsp70* intergenic regions

Consensus bases are highlighted in black, similar bases are highlighted in gray. Asterisk (*) indicates the only perfect trimeric site. In the consensus, bold/underlined bases are predicted to bind HSF (Perisic et al. 1989).

Figure 2. Activity of *AaHsp70* putative promoter constructs in *Aedes aegypti* cells and embryos

The parent structure of *AaHsp70* inverted gene pairs are shown above each of the test constructs, with solid block arrows indicating each *hsp70* ORF and open block arrows indicating the FFLuc ORF. Line arrows indicate the start of transcription (0), with the number of nucleotides upstream (-) or downstream (+) indicated below each vertical dotted line. White vertical bars indicate the approximate locations of HSE clusters, with numerals above displaying the size of HSE clusters when different from 3. For both *AaHsp70Aa* (**A**) and *AaHsp70Bb* (**B**), x-axis indicates the ratio of FFLuc to RLuc. Heat shocked (HS) and control (C) groups are indicated for each putative promoter construct. For cells, each data point represents a single well of a 12-well plate. Statistical significance between control and heat shock values was determined using an unpaired t-test; Welch's correction was used where variances were found to be unequal. For embryos, each data point represents a group of 80-120 injected individuals; data were rank-transformed and significance was determined using the Mann-Whitney test. Significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) is indicated. For comparisons between groups, Tukey's Honestly Significant Difference (HSD) test was used based on heat-shock values only; values not connected by the same letter are statistically independent groups. We note that the magnitude of the ratio of FFLuc to RLuc is not directly comparable between cell and embryo data due to the different temperatures used for heat shock as well as different plasmid ratios (see Experimental Procedures).

Figure 3. *AaHsp70* promoter activity in adult female transgenic *Aedes aegypti*
AaHsp70Aa-1373-FFluc and *AaHsp70Bb*-1456-FFluc transformed adult females were heat shocked (HS) and FFluc values were determined after a 1 hr recovery period and compared with untreated control (C) siblings. Y-axis indicates the log₁₀-transformed value of the total number of light units, with each data point representing a single adult. The Mann-Whitney test was used on rank-transformed data, with significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) indicated above each bar. Numbers indicate the fold induction as determined by dividing the mean heat-shock value by the mean control value.

Figure 4. Transcript abundance and luciferase activity over time in transgenic *Aedes aegypti* following heat shock

(A) Northern analysis of FFLuc or *hsp70* transcripts following heat shock of transgenic strain *AaHsp70Aa*-FFLuc #P10. Exposures were standardized based on the specific activity of each probe; ethidium bromide stained rRNA loading controls are shown below each blot. (B-C) Luciferase activity was measured over time for *AaHsp70Aa*-1373-FFLuc line #P10 (B) or *AaHsp70Bb*-1456-FFLuc line #P6 (C) adult females following heat shock for 1 hour at 39°C. Fold-changes in luciferase activity compared to the non-heat shocked control (c) are labeled and values not connected by the same letter are significantly different according to Tukey's HSD.

Figure 5. *AaHsp70* promoter activity in transgenic *Ae. aegypti* tissues

Firefly luciferase activity was measured at 1-4 h post heat shock for heads (A), midguts (B), salivary glands (C) and ovaries (D) dissected from transformed individuals. Y-axis indicates the $\log_{10}$ -transformed value of the total number of light units, with each data point representing a pool of 10. The Mann-Whitney test was used on rank-transformed data, with significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) indicated above each bar. Numbers indicate the fold induction as determined by dividing the mean heat-shock value by the mean control value.

Table 1
***AaHsp70* intergenic regions are enriched for HSEs**

Intergenic region	Length (bp)	# HSE	Expected (sd) ^a	z score (P value)
<i>2P tail-tail (nTTCn-nGAAn-nnnnn)</i>				
<i>AaHsp70Aa-Ab</i>	2628	2	0.8 (+/- 0.8)	1.6 (0.057)
<i>AaHsp70Ba-Bb</i>	3071	2	1.3 (+/-1.0)	0.7 (0.242)
<i>AaHsp70Ca-Cb</i>	1077	2	0.4 (+/-0.7)	2.3 (0.010)
<i>2P head-head (nGAAn-nTTCn-nnnnn)</i>				
<i>AaHsp70Aa-Ab</i>	2628	7	1.1 (+/- 1.1)	5.5 (<0.0001)
<i>AaHsp70Ba-Bb</i>	3071	6	1.0 (+/-0.9)	5.3 (<0.0001)
<i>AaHsp70Ca-Cb</i>	1077	3	0.4 (+/-0.6)	4.7 (<0.0001)
<i>Step/Gap Type (nTTCn-nnnnn-nTTCn)</i>				
<i>AaHsp70Aa-Ab</i>	2628	15	1.8 (+/- 1.3)	9.9 (<0.0001)
<i>AaHsp70Ba-Bb</i>	3071	8	2.0 (+/- 1.2)	4.8 (<0.0001)
<i>AaHsp70Ca-Cb</i>	1077	5	0.9 (+/- 1.1)	3.7 (<0.0001)

^a Mean number of HSE elements as expected by chance based on 30 shuffled versions of the parent sequence (Stothard 2000); sd indicates one standard deviation.