Published in final edited form as:

J Med Entomol. 2009 May ; 46(3): 496–504.

Identification and Characterization of Heat Shock 70 Genes in *Aedes aegypti* (Diptera: Culicidae)

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Abstract

Heat shock genes are highly evolutionarily conserved and are expressed to varying degrees in all organisms in response to stress. Heat shock 70 (*hsp70*) genes have been well characterized in a number of organisms, most notably *Drosophila melanogaster*, but not as yet for any of the major arthropod-borne viral mosquito vectors. To identify *hsp70* genes in the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae), basic local alignment searches of the *Ae. aegypti* genome were performed using *D. melanogaster* Hsp70 protein sequences as query. Two clusters of six previously unannotated *AaHsp70* genes were identified and found to be organized into three pairs of nearly identical open reading frames, which mapped to two genomic scaffolds. Consistent with a designation as heat shock genes, no detectable level of expression of *AaHsp70* genes was observed under normal rearing conditions (28°C), with robust expression observed with a heat shock of 37–39°C. Northern analysis showed heat-inducible expression of putative *AaHsp70* genes at all life stages and in all tissues tested in a time- and temperature-dependent manner. Monitoring of *AaHsp70* gene expression levels in field-caught *Ae. aegypti* may serve as a general marker for stress. In addition, promoter sequences from *AaHsp70* genes may be used to control the expression of transgenes in an inducible manner.

Keywords

heat shock protein 70; *Aedes aegypti*; heat shock

Aedes aegypti is a significant vector of disease agents, capable of transmitting the viruses that cause yellow fever, dengue fever, and the more severe dengue hemorrhagic fever (Tatem et al. 2006). According to the WHO (2008), 2.5 billion people are at risk from dengue, with 50 million dengue infections worldwide each year. In 2007 in the Americas alone, there were 890,000 dengue infections, 26,000 of which were dengue hemorrhagic fever cases (WHO 2008).

Aedes aegypti is one of the most highly studied arthropods, not only for its importance as a vector of disease but also because of its ease to rear in the laboratory (Severson et al. 2004). With sequence data available for both *Ae. aegypti* and *Anopheles gambiae*, comparative genomics is an important tool for understanding vector biology. Comparative analysis with *Drosophila melanogaster* will also lead to better understanding of each of the mosquito genomes (Waterhouse et al. 2008).

Heat shock protein 70 (*Hsp70*) genes are members of a highly conserved gene family, with proteins related to *D. melanogaster hsp70* found in prokaryotes and higher and lower

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eukaryotes (Craig 1985, Mukhopadhyay et al. 2003). The chaperone proteins encoded by these genes are responsible for preventing the cytotoxic aggregation of denatured proteins, as well as for the refolding of denatured proteins (Bettencourt and Feder 2002). Expression of *hsp70* genes is induced by various stresses including heat, anoxia, and exposure to ethanol and chemical treatments including sodium arsenite, cadmium chloride, and sodium salicylate (Craig 1985, Tanguay 1988, Li and Werb 1982, Liu et al. 1994). *D. melanogaster hsp70* genes are well characterized and serve as a reference for comparative genomics studies of *hsp70* genes of other organisms (Severson et al. 2004, Waterhouse et al. 2008).

DmHsp70 promoter sequences are commonly used to drive the expression of exogenous gene products, to induce transposition events, and also for gene function studies in *D. melanogaster* (Golic and Lindquist 1989, Wimmer 2003) and heterologous systems such as *B. mori* (Uhlirova et al. 2002, Dai et al. 2007). The *DmHsp70* promoter has also been shown to drive transgene expression in cultured mosquito cells (Zhao and Eggleston 1999). However, for some applications, an endogenously derived promoter may prove to be more useful than a heterologous promoter, particularly with respect to genetically modified mosquito strategies where limiting the amount of non-native DNA is critical.

Aedes aegypti is the best-characterized species within the subfamily Culicinae (Nene et al. 2007). A joint sequencing effort between the Broad Institute and The Institute for Genomic Research (TIGR) resulted in eight-folds coverage of the ≈ 1.3 -Gb Liver-pool genome (Lobo et al. 2007, Nene et al. 2007). Before the completion of the *Ae. aegypti* genome sequencing project, a putative *AaHsp70* gene was used by Fulton et al. (2001) as a marker for single-strand conformation polymorphism (SSCP) analysis based on an unpublished submission to GenBank. Isoe et al. (2007) also identified a putative *AaHsp70* gene. This gene, however, was found not to be heat inducible (Isoe et al. 2007), suggesting that it may be a heat shock cognate instead. Heat shock cognates are closely related to heat shock proteins but are constitutively expressed and are not part of the stress response pathway. Last, a gene identified as *hsp70* was annotated on genomic scaffold 1.116 and mapped to the p-arm of chromosome 3 (Nene et al. 2007). However, uncertainty remains as to the nature and accuracy of these preliminary annotations. In this paper, we definitively identify and describe the expression pattern of novel *hsp70* genes from *Ae. aegypti*. We also present evidence that all three previous reports of *AaHsp70* genes were actually descriptions of a heat shock cognate gene orthologous to *D. melanogaster Hsc70-4*.

Materials and Methods

Mosquito Strains and Rearing

Aedes aegypti (Liverpool strain) mosquitoes were maintained at 28°C and 80% RH with a photoperiod of 16 h of light and 8 h of darkness. Larvae were fed pulverized fish food and reared ≈ 300 per pan in 4 liters of reverse osmosis (RO) purified water until pupation. Adult mosquitoes were maintained on sucrose and were blood fed using artificial membrane feeders and defibrinated sheep blood (Colorado Serum Company, Denver, CO).

Amplification and Cloning of *AaHsp70* Genes

AaHsp70 genes were amplified from Liverpool strain genomic DNA using Platinum *Pfx* polymerase chain reaction (PCR; Invitrogen, Carlsbad, CA) ($2\times$ *Pfx* amplification buffer, 0.3 mM dNTPs, 1 mM MgSO₄, 0.3 μ M primers, $1\times$ enhancer solution, 1 U Platinum *Pfx* DNA polymerase). Genomic DNA was digested with either *Sac*II or *Bgl*II to separate inverted *hsp70* gene pairs. All attempts to amplify *hsp70* genes from undigested mosquito DNA were unsuccessful, presumably because the nearly identical open reading frames and 5' regions would be expected to form hairpin structures during the amplification process. Gene regions

for putative *AaHsp70Ba*, *-Bb*, *-Ca*, and *-Cb* were amplified using *Sac*II digested genomic DNA from male *Ae. aegypti* (94°C, 5 min; 94°C, 30 s, 60°C, 1 min, 68°C, 3 min, 35 cycles; 68°C, 10 min), and putative *AaHsp70Aa* and *-Ab* were amplified using *Bgl*III digested genomic DNA from male *Ae. aegypti*. Amplified *AaHsp70* genes were cloned using *Kpn*I restriction sites added to the primers used for the original amplification. Primers used to amplify *AaHsp70* gene regions are listed in Table 1. Plasmids containing open reading frames from each gene were sequenced using ABI BigDye Terminators v 3.0 (Foster City, CA).

Mosquito Heat Shock Regimens

Groups of adult female *Ae. aegypti* mosquitoes ($n = 35\text{--}40$) were heat shocked in a prewarmed oven $\approx 3\text{--}5$ d after eclosion. Each heat shock lasted 1 h at a predetermined temperature ranging from 35 to 41°C in 2°C increments, with $\approx 80\%$ humidity (35 ± 0.3 , 37 ± 0.6 , 39 ± 0.8 , and $41 \pm 0.4^\circ\text{C}$). Mosquitoes were permitted to rest at 28°C for varying amounts of time ranging from 30 min to 24 h after the heat shock regimen. Once the predetermined resting period was over, mosquitoes were harvested into microcentrifuge tubes and snap frozen in liquid nitrogen to preserve RNA. Third- and fourth-instar larvae and pupae were heat shocked using a 37°C water bath for 1 h and were permitted to rest for 30 min before being snap frozen. Groups of adult males were heat shocked for 1 h at 39°C and allowed to rest for 30 min before being snap frozen. The temperature during heat shock was monitored and verified using a HOBO data logger (Onset Computer, Bourne, MA).

Northern Analysis and Rapid Amplification of cDNA Ends

Total RNA (5 μg) from each experimental group was electrophoresed in a 1.2% agarose, 1 $\times$ MOPS (0.023 M 3-morpholinopropane sulfonic acid, 0.3 mM NaOAc, 0.2 mM EDTA), and 2% formaldehyde gel at 90 V. RNA was blotted onto a positively charged nylon membrane (Immobilon-NY⁺; Millipore, Concord, MA). Blots were prehybridized at 65°C in prewarmed Church's buffer (0.25 M sodium phosphate buffer, 1 mM EDTA, 7% SDS) using a Fisher Isotemp hybridization oven (Fisher, Pittsburgh, PA). Random primed probes were labeled with [α -³²P]dATP (specific activity 3000 Ci/mmol) using the Amersham Megaprime DNA Labeling System (GE Healthcare, Buckinghamshire, United Kingdom). The specific activity of the probes was determined using a Beckman-Coulter LS6500 Multi-purpose Scintillation Counter. Probes were purified using illustra NICK columns (GE Healthcare) and added to prewarmed Church's buffer to hybridize overnight at 65°C. Blots were washed twice with 2 $\times$ saline-sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) for 20 min each at 65°C and twice with 0.2 $\times$ SSC (0.03 M sodium chloride, 0.003 M sodium citrate) and 0.1% SDS for 20 min each at 65°C.

DNA templates to be radiolabeled as probes for Northern analysis were generated from putative 3'-untranslated regions (UTRs) of *AaHsp70* gene paralogs and from sequence common to all 12 putative *AaHsp70* genes. The common probe was amplified using One Step RT-PCR (50°C, 30 min, 95°C, 15 min; 94°C, 30 s, 60°C, 30 s, 72°C, 1 min, 35 cycles; 72°C, 10 min) using primers (5'-TTGGTTGATGTGGCTCCACTCTCATTGG-3' and 5'-TTGTGCTCGAACTCGTCCTTCTCG-3') and optional Q solution (Qiagen, Valencia, CA). To generate DNA fragments specific for pairs of *AaHsp70* paralogs, a portion of each putative 3' UTR was amplified using One Step RT-PCR and the following primers: *AaHsp70Aa* (5'-AAGTTGACTAAATTGAGTTGAGATACGAGACTGAATGAG-3' and 5'-TTGTTATAGTTTATTTTCGTGAAAACATTCTACTTATGATTAC-3'), *AaHsp70Ab* (5'-AGGAGAAGTGAAATGAGACTGAATGTTTTAGTAGAG-3' and 5'-AACCTTATTCTCTAAGGCTTATGTCAGCAATTCC-3'), *AaHsp70Ba* (5'-AATTGGGTTGACAAACGAGACTGAATGAG-3' and 5'-TACAACGATAATAATTGCAAATACGATCTACGAATCC-3'), *AaHsp70Bb* (5'-TTGAGGAAGTCGACTAAAGCGAATGGAGAGG-3' and 5'-

TTCGTA AAAACAAGCTGTACACATTAAATAACTTTCTAAC-3'), *AaHsp70Ca* (5'-TTGAGGAAGTCGACTAAAGTGAATGGAGCG-3' and 5'-AACAAGCTCAACATAATAGGAACATTAACAACTTCC-3'), and *AaHsp70Cb* (5'-TTGAGGAAGTGGACTAAGTATATCAAGGCATTTAAACCC-3' and 5'-AAGCATTAGCTTGAGTCGTCAATCTTTAAGATTACTG-3'). Gene fragments were cloned using the same oligonucleotide primers with added *KpnI* recognition sites in the same manner as the open reading frames. To generate cDNA for use in rapid amplification of cDNA ends (RACE) reactions, the Promega PolyAT-tract mRNA Isolation System was used to purify total RNA extracted from 60 female Liverpool *Ae. aegypti*. To perform 5' and 3' RACE, the Clontech (Mountain View, CA) SMART RACE amplification kit was used with gene-specific primers: for 5' RACE (5'-CGATTTTCCTTGGTCGTTGGCGATGATTTC-3', 5'-CGATTTCCCTGGTCGTTGGCGATGATTTC-3', 5'-CGATTTCCCTGGTCGTTGGCAATGATTTC-3') and for 3' RACE *AaHsp70Aa* (5'-TGCGGTACAAGCTGCCATCCTCAGTGGAGAC-3'), *AaHsp70Ab* (5'-CGATCATGACTCGATTGCATCAGGGTGG-3'), *AaHsp70Ba* (5'-ACGAAAAGCAACGCGAACGTGTCTCTGCC-3'), *AaHsp70Bb* (5'-CGATCATGACTCGTTTGCATCAAGGTGGAG-3'), *AaHsp70Ca* (5'-TTCGAGCACAAGATGCAAGAGCTGAGTCG-3'), and *AaHsp70Cb* (5'-ATCAGCTGGCCAGCAAGGAGGAAATGGACC-3').

Phylogenetic Analysis

AaHsp70 gene sequences have been deposited in GenBank and are available using accession numbers FJ177309-14 and are listed in Table 2. Other sequences used for phylogenetic analysis include *Anopheles gambiae Hsp70* genes AGAP004582 and AGAP012891, as well as *AgHsc70B*, ENSANGP00000017398; and *D. melanogaster* genes *Hsp70Aa*, CG31366; *Hsp70Ab*, CG18743; *Hsp70Ba*, CG31449; *Hsp70Bb*, CG31359; *Hsp70Bbb*, CG5834; *Hsp70Bc*, CG6489; and *Hsc70-4*, CG4264 (Sim et al. 2007). ClustalW parameters used to align *Hsp70* protein sequences were pairwise gap opening penalty of 10, pairwise gap extension penalty of 0.1, multiple gap opening penalty of 10, and multiple gap extension penalty of 0.2 with negative matrix. A linearized neighbor-joining tree was produced using MEGA 4.1 (Tamura et al. 2007).

Results

AaHsp70 Gene Organization

To identify *hsp70* genes in *Ae. aegypti*, we performed tBLASTn searches of the *Ae. aegypti* genomic scaffolds using the *D. melanogaster Hsp70Aa* protein sequence. Eighteen putative genes were identified with an expected cut-off value of 0 (Table 2). The predicted protein sequences of 17 of the 18 genes (one sequence contained major gaps that prevented in silico translation) were used to back-query the *D. melanogaster* gene set, and the best match for each is reported in Table 2. Of the 18 genes, 12 were a best match for *D. melanogaster hsp70*. Six of the putative *AaHsp70* genes mapped to genomic supercontig 1.680 (Table 2; Fig. 1), located on chromosome 1 (Nene et al. 2007), with a second cluster of six putative *AaHsp70* genes on the unmaped supercontig 1.824 (Table 2; Fig. 1).

Putative *AaHsp70* protein sequences were compared with *An. gambiae* (*AgHsp70*) and *D. melanogaster* (*DmHsp70*) genes and cognates using ClustalW and a neighbor-joining tree was produced (Fig. 2). The putative *AaHsp70* genes formed an independent set of clades from *An. gambiae* and *D. melanogaster hsp70* genes, consistent with the notion that *hsp70* genes have undergone several independent duplication events in each of these organisms. To determine whether the sequences previously annotated as *AaHsp70* (Fulton et al. 2001, Isoe et al. 2007) corresponded to the same gene sequences we describe here, or to a novel set of genes, we

performed BLASTn searches using GenBank deposited nucleotide sequences as a query against the *Ae. aegypti* genomic supercontigs (Nene et al. 2007). Both DQ453756 (Isoe et al. 2007) and AI658418 (Fulton et al. 2001) were found to map to genomic supercontig 1.116 (positions 1031943–1032601 and 1032458–1035831, respectively). An open reading frame was detected in this region and the predicted protein sequence was used as a query to search the *An. gambiae* and *D. melanogaster* genomes using tBLASTn. As shown in Table 2 and Fig. 2, the heat shock–like gene identified on supercontig 1.116 is most closely related to *AgHsc70B* and *DmHsc70–4*, suggesting that the most appropriate annotation for this gene is *AaHsc70–4*.

As the two clusters of six *AaHsp70* genes were near identical to each other, we focused the rest of our analysis on the cluster located on scaffold 1.680, where the sequence assembly was of higher quality with fewer gaps. To document the exact start and stop of transcription of *AaHsp70* transcripts, we performed RACE. Ninety-five percent of 5' RACE clones (35/37 clones) supported a consistent start of transcription, which was found to be 182 bases upstream of the start of translation for *AaHsp70Aa* and *AaHsp70Cb*, 181 bases upstream for *AaHsp70Ba*, *AaHsp70Bb*, and *AaHsp70Ca*, and 180 bases upstream for *AaHsp70Ab*. Multiple 3' RACE products were sequenced for each *AaHsp70* paralog, with the longest most abundant clone assumed to represent the end of transcription. The 3'-UTRs were found to be far more variable in length than the 5' region, with *AaHsp70Ab* (14/21 clones) being the shortest (135 nt 3' UTR), followed by *AaHsp70Ca* (4/7 clones, 145 nt), *AaHsp70Aa* (5/8 clones, 150 nt), *AaHsp70Bb* (6/11 clones, 150 nt), *AaHsp70Ba* (3/4 clones, 208 nt), and *AaHsp70Cb* (9/9 clones, 282 nt). Each of the six putative open reading frames was nearly identical in length, with *AaHsp70Aa*, *-Ab*, *-Ba*, and *-Bb* at 1,914 nt; *AaHsp70Ca* at 1,911 nt; and *AaHsp70Cb* at 1,908 nt. To better resolve the within-cluster phylogenetic relationship of *AaHsp70* genes, Geneious software (Biomatters, Auckland, New Zealand) was used to produce a ClustalW alignment of putative *AaHsp70* gene transcripts (Fig. 3). Open reading frames of *AaHsp70* genes were found to be 77–96% identical at the nucleotide level, with *AaHsp70Cb* being the most divergent. The 5' untranslated regions of putative *AaHsp70* genes were found to share 88% nucleotide identity, whereas the 3'-UTRs share only 38% identity. Pairwise comparisons between putative *AaHsp70* genes showed that *AaHsp70Bb* and *AaHsp70Ca* were most similar, with >95% identity at the nucleotide level (Table 3). No other strong pairwise relationship was evident as *AaHsp70Aa*, *AaHsp70Ab*, and *AaHsp70Ba* were essentially equally similar to each other (93.5%), whereas *AaHsp70Cb* was equivalently divergent from the other five genes (Table 3). This is in agreement with the protein alignment presented in Fig. 1, where three *AaHsp70* clades were also identified (*Aa*, *Ab*, and *Ba*; *Bb* and *Ca*; and *Cb*).

Expression of *AaHsp70* Genes

To confirm our bioinformatic annotation of this cluster as true heat shock genes, we next sought to determine whether mRNA expression could be induced through exposure to heat shock. Female *Ae. aegypti* were exposed to heat shock regimens with temperatures ranging from 35 to 41°C in 2°C increments for 1 h. Total RNA was harvested at various times after exposure to heat shock and was subject to Northern analysis using a probe capable of hybridizing with putative *AaHsp70* genes but not the closely related heat shock cognates (Fig. 4). Expression of *AaHsp70* mRNAs was induced to various extents in all heat shock regimens, whereas no expression was detected in control mosquitoes not subjected to heat shock (Fig. 4). The highest levels of expression were observed after exposure to 39°C, with only modest induction occurring at 35°C and heat shock transcripts detected only up to 2 h after heat shock. After heat shock at 37°C, *AaHsp70* transcripts were detected up to 24 h, although expression did not match that seen at 39°C. *AaHsp70* expression at 41°C could not be documented because all mosquitoes died during the 1-h heat shock regimen.

Multiple copies of genes with the same function can indicate inactive evolutionary remnants (Ingolia et al. 1980). Thus, it was possible that not all of the *AaHsp70* genes we identified were still active or responded to heat shock identically. Indeed, at least one of the *AaHsp70* genes (*Bb'*) contained deleterious frame-shifts. To document the expression levels of each putative *AaHsp70* gene, we repeated our heat shock regimen with female *Ae. aegypti* heat shocked at 39°C for 1 h. After a rest period of 0–4 h, total RNA was extracted and Northern analysis was performed using six paralog-specific probes derived from the unique 3' UTR of each gene. Of the six putative *AaHsp70* genes we examined, at least five showed detectable expression by this method, with only *AaHsp70Ab* failing to produce a detectable hybridization signal (Fig. 5). The highest levels of expression were observed for *AaHsp70Aa*, *AaHsp70Ba*, and *AaHsp70Ca* transcripts, with weak expression seen for *AaHsp70Cb* (Fig. 5).

In *D. melanogaster*, *hsp70* genes are expressed ubiquitously throughout the body of the fly after heat shock. To document any tissue specificity of putative *AaHsp70* genes, RNA from heat-shocked *Ae. aegypti* heads, salivary glands, thoraxes lacking salivary glands, ovaries, midguts, as well as from heat-shocked larvae, pupae, and males was subject to Northern analysis using a probe capable of hybridizing with *AaHsp70* genes but not the closely related cognates. As expected, robust expression was observed after heat shock in all tissues tested (Fig. 6A), in all life stages, and in male mosquitoes (Fig. 6B).

Discussion

The basic arrangement of *hsp70* genes in *Ae. aegypti* closely resembles that of *D. melanogaster* (Gong and Golic 2004) and *An. albimanus* (Benedict et al. 1993), with a pair of nearly identical open reading frames organized as an inverted pair. We have successfully identified two clusters of three such pairs in close proximity in the *Ae. aegypti* genome. Although the presence of these two clusters of six *AaHsp70* genes would indicate that this mosquito has up to 12 *hsp70* paralogs, the fragmented nature of the genomic assembly does not allow us to rule out the possibility that these two clusters are allelic variants. Phylogenetic analysis of *AaHsp70* genes compared with *hsp70* genes from *D. melanogaster* and *An. gambiae* showed a pattern of divergence in which both *Ae. aegypti* and *An. gambiae* genes diverged from *D. melanogaster* before duplication of the ancestral inverted pair within each species, as predicted by Benedict et al. (1993). *DmHsp70* genes are grouped at two loci on the right arm of chromosome 3 with the first inverted pair separated by ≈ 1.7 Kb, followed by three or four genes at the second locus organized with one gene proximal to the centromere separated by ≈ 40 Kb from two or three tandem genes of inverted polarity (Craig 1985, Gong and Golic 2004). It is believed that a duplication of the evolutionarily ancient, highly stable two gene cluster lead to the current organization of *DmHsp70* genes, with tandem duplications leading to the fifth and sixth genes allowing *D. melanogaster* greater thermotolerance and niche expansion (Bettencourt and Feder 2001). It is possible that multiple copies of *AaHsp70* may also contribute to the distribution and heat tolerance of *Ae. aegypti*. *DmHsp70* genes are separated by large amounts of repetitive DNA resulting from transposable S-elements, and these are thought to have played a role in the duplication of heat shock genes from two to four in *Drosophila* (Bettencourt and Feder 2001, Evgen'ev et al. 2004, Gong and Golic 2004). The *Ae. aegypti* genome is highly repetitive, with $\approx 70\%$ of the genome being transposable elements/repetitive DNA (Nene et al. 2007). At least two duplication events of the ancestral *hsp70* gene two gene pair seem to have occurred in *Ae. aegypti* after divergence from both *D. melanogaster* and *An. gambiae*, and these may have occurred by similar mechanisms and for similar reasons. Unlike *Drosophila*, however, whose two pairs of heat shock genes are separated by >500 kb (Evgen'ev et al. 2004), and *An. albimanus*, whose two pairs are separated by ≈ 20 cM (Benedict et al. 1993), all three pairs of *AaHsp70* genes were spaced within a span of 42 kb. Because the scaffold 1.824 is unmapped, the spacing between the two clusters of *AaHsp70* genes remains unknown. In addition, unlike *hsp70* genes described in *An. albimanus*,

Ae. aegypti *hsp70* genes were not found to be more closely related within each inverted pair compared with between pairs (Benedict et al. 1993). It is possible that the differences we observed between the *AaHsp70* genes are the result of gene inactivation followed by drift rather than being a strict footprint of inheritance. Ultimately, we cannot draw firm conclusions at this point as to how the ancestral two-gene pair evolved into the six gene clusters we observed. Future studies of *hsp70* genes from other members of the genus *Aedes* would likely provide additional insight.

The putative *Ae. aegypti* heat shock cognate identified as *AaHsp70* by Isoe et al. (2007) was mapped to supercontig 1.116 on chromosome 3 (Nene et al. 2007). However, when we compared this sequence to the *D. melanogaster* genome, the best match is actually a heat shock cognate, a closely related gene that is constitutively expressed and not heat inducible. As shown in Fig. 2, the cognate identified by Isoe et al. (2007) forms a separate clade with cognates from both *D. melanogaster* and *An. gambiae*, further supporting the idea that this gene may be a heat shock cognate. Gene annotations are often the result of predictions based on homology before complete genome sequencing. However, examples such as this underscore the importance of confirming such predictions before experimentation.

To balance the benefits of *hsp70* gene expression under stress conditions with detrimental effects of expression under normal conditions, *DmHsp70* genes have been found to be self-regulating (Craig 1985, Lindquist 1986). *DmHsp70* genes are targeted for degradation by signals in the 3'-UTRs, and under normal conditions, *hsp70* transcripts are rapidly degraded (Petersen and Lindquist 1989, Feder et al. 1992). In cultured *D. melanogaster* cells, induced expression of *DmHsp70* genes under normal conditions was deleterious to cells and resulted in a decreased growth rate (Solomon et al. 1991, Feder et al. 1992). Extreme temperatures induce long-lasting synthesis and very stable *DmHsp70* mRNA, whereas at lower temperatures, expression proceeds until heat shock proteins have accumulated to a level proportional to the severity of heat shock before further transcription is repressed (Lindquist 1986, Nover 1991). *AaHsp70* genes were found to exhibit a similar expression pattern, since under extreme heat shock conditions (39°C) high levels of expression are initiated rapidly and transcript was detected up to 24 h after heat shock, whereas expression of *hsp70* transcripts was less robust and short lived under a more modest heat shock of 35°C (Fig. 4).

Induced mRNA expression levels were found to vary among the *AaHsp70* paralogs we examined. Interestingly, the first gene of each inverted pair seemed to be expressed preferentially over the second (see Figs. 1 and 5), with *AaHsp70Aa* > *AaHsp70Ab*, *AaHsp70Ba* > *AaHsp70Bb* and *AaHsp70Ca* > *AaHsp70Cb*. Also, this expression seemed to be proportional, so that the highest/lowest level of expression was observed from the *AaHsp70Aa/Ab* pair and the next highest/lowest expression from *AaHsp70Ca/Cb*, followed by *AaHsp70Ba/Bb*, which had almost equivalent expression between the two genes. The reasons for this are currently unknown, but may be related to the positioning of replication complexes during transcriptional pausing. In *D. melanogaster*, RNA polymerase II is constantly bound upstream of the start of transcription to maintain an open chromatin configuration (Karpov et al. 1984, Craig 1985) because heat shock genes must be available for near immediate action under stress conditions. This configuration is likely to occur in *Ae. aegypti* as well and is consistent with the observation of high levels of *AaHsp70* expression detected immediately after 37°C and 39°C heat shocks. Although we could not detect *AaHsp70* Ab expression by Northern analysis, RACE transcripts were recovered for this gene, indicating some level of transcription.

AaHsp70 genes are expressed in all life stages and in all tissues tested in response to heat shock. Therefore, isolating heat inducible *AaHsp70* promoter elements would be valuable for transgenesis and gene function studies, particularly when it is important to minimize the

presence of exogenous sequences. Further studies of *AaHsp70* regulatory regions would make such studies possible in *Ae. aegypti*. *Hsp70* gene expression also serves as a marker for stress, and monitoring levels of *AaHsp70* gene expression in wild populations could serve as a physiological indicator at the population level.

Acknowledgments

We thank M. Anderson, J. Overcash, M. Wiley, and B. Traver and other members of the Myles/Adelman laboratories for technical assistance. This project was supported by NIAID Grant R21AI071208-01 and by Virginia Tech startup funds to Z.N.A.

References Cited

- Benedict MQ, Cockburn AF, Seawright JA. The Hsp70 heat-shock gene family of the mosquito *Anopheles albimanus*. *Insect Mol Biol* 1993;2:93–102. [PubMed: 9087548]
- Bettencourt BR, Feder ME. Hsp70 duplication in the *Drosophila melanogaster* species group: how and when did two become five? *Molec Biol Evol* 2001;18:1272–1282. [PubMed: 11420366]
- Bettencourt BR, Feder ME. Rapid concerted evolution via gene conversion at the *Drosophila* hsp70 genes. *J Molec Evol* 2002;54:569–586. [PubMed: 11965431]
- Craig EA. The heat shock response. *CRC Crit Rev Biochem* 1985;18:239–280. [PubMed: 2412760]
- Dai H, Jiang R, Wang J, Xu G, Cao M, Wang Z, Fei J. Development of a heat shock inducible and inheritable RNAi system in silkworm. *Biomolec Eng* 2007;24:625–630.
- Evgen'ev MB, Zatsepina OG, Garbuz D, Lerman DN, Velikodvorskaya V, Zelentsova E, Feder ME. Evolution and arrangement of the hsp70 gene cluster in two closely related species of the virilis group of *Drosophila*. *Chromosoma* 2004;113:223–232. [PubMed: 15480729]
- Feder JH, Rossi JM, Solomon J, Solomon N, Lindquist S. The consequences of expressing Hsp70 in *Drosophila* cells at normal temperatures. *Genes Dev* 1992;6:1402–1413. [PubMed: 1644286]
- Fulton RE, Salasek ML, DuTeau NM, Black WCT. SSCP analysis of cDNA markers provides a dense linkage map of the *Aedes aegypti* genome. *Genetics* 2001;158:715–726. [PubMed: 11404335]
- Golic KG, Lindquist S. The F1p recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell* 1989;59:499–509. [PubMed: 2509077]
- Gong WJ, Golic KG. Genomic deletions of the *Drosophila melanogaster* Hsp70 genes. *Genetics* 2004;168:1467–1476. [PubMed: 15579699]
- Ingolia TD, Craig EA, McCarthy BJ. Sequence of three copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. *Cell* 1980;21:669–679. [PubMed: 6777045]
- Isoe J, Kunz S, Manhart C, Wells MA, Miesfeld RL. Regulated expression of microinjected DNA in adult *Aedes aegypti* mosquitoes. *Insect Mol Biol* 2007;16:83–92. [PubMed: 17257211]
- Karpov VL, Preobrazhenskaya OV, Mirzabekov AD. Chromatin structure of Hsp-70 genes, activated by heat-shock—selective removal of histones from the coding region and their absence from the 5' region. *Cell* 1984;36:423–431. [PubMed: 6420075]
- Li GC, Werb Z. Correlation between synthesis of heat-shock proteins and development of thermotolerance in Chinese-hamster fibroblasts. *Proc Natl Acad Sci USA* 1982;79:3218–3222. [PubMed: 6954473]
- Lindquist S. The heat-shock response. *Annu Rev Biochem* 1986;55:1151–1191. [PubMed: 2427013]
- Liu RY, Corry PM, Lee YJ. Regulation of chemical stress-induced hsp70 gene expression in murine L929 cells. *J Cell Sci* 1994;107:2209–2214. [PubMed: 7983179]
- Lobo NF, Campbell KS, Thaner D, Debruyne B, Koo H, Gelbart WM, Loftus BJ, Severson DW, Collins FH. Analysis of 14 BAC sequences from the *Aedes aegypti* genome: a benchmark for genome annotation and assembly. *Genome Biol* 2007;8:R88. [PubMed: 17519023]
- Mukhopadhyay I, Saxena DK, Chowdhuri DK. Hazardous effects of effluent from the chrome plating industry: 70 kDa heat shock protein expression as a marker of cellular damage in transgenic *Drosophila melanogaster* (hsp70-lacZ). *Environ Health Perspect* 2003;111:1926–1932. [PubMed: 14644668]

- Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi ZY, Megy K, Grabherr M, et al. Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science* 2007;316:1718–1723. [PubMed: 17510324]
- Nover, L. Heat shock response. CRC: Boca Raton, FL; 1991.
- Petersen RB, Lindquist S. Regulation of Hsp70 synthesis by messenger-RNA degradation. *Cell Regul* 1989;1:135–149. [PubMed: 2519613]
- Severson DW, Knudson DL, Soares MB, Loftus BJ. *Aedes aegypti* genomics. *Insect Biochem Mol Biol* 2004;34:715–721. [PubMed: 15242713]
- Sim C, Hong YS, Tsetsarkin KA, Vanlandingham DL, Higgs S, Collins FH. *Anopheles gambiae* heat shock protein cognate 70B impedes o'nyong-nyong virus replication. *BMC Genom* 2007;8:231–242.
- Solomon JM, Rossi JM, Golic K, McGarry T, Lindquist S. Changes in Hsp70 alter thermotolerance and heat-shock regulation in *Drosophila*. *New Biologist* 1991;3:1106–1120. [PubMed: 1777484]
- Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molec Biol Evol* 2007;24:1596–1599. [PubMed: 17488738]
- Tanguay RM. Transcriptional activation of heat-shock genes in eukaryotes. *Biochem Cell Biol Biochim Biol Cell* 1988;66:584–593.
- Tatem AJ, Hay SI, Rogers DJ. Global traffic and disease vector dispersal. *Proc Natl Acad Sci USA* 2006;103:6242–6247. [PubMed: 16606847]
- Uhlirova M, Asahina M, Riddiford LM, Jindra M. Heat-inducible transgenic expression in the silk-moth *Bombyx mori*. *Dev Genes Evol* 2002;212:145–151. [PubMed: 11976953]
- Waterhouse RM, Wyder S, Zdobnov EM. The *Aedes aegypti* genome: a comparative perspective. *Insect Mol Biol* 2008;17:1–8. [PubMed: 18237279]
- [WHO] World Health Organization. Dengue and dengue haemorrhagic fever, fact sheets. World Health Organization; Geneva, Switzerland: 2008.
- Wimmer EA. Innovations: applications of insect transgenesis. *Nat Rev Genet* 2003;4:225–232. [PubMed: 12610527]
- Zhao YG, Eggleston P. Comparative analysis of promoters for transient gene expression in cultured mosquito cells. *Insect Mol Biol* 1999;8:31–38. [PubMed: 9927172]

Fig. 1.

Genome organization and relationship of *AaHsp70* genes. Arrangement of *AaHsp70* genes in *Ae. aegypti*. *AaHsp70* genes are indicated by solid arrows. Lines under each gene indicate areas reamplified, cloned, and sequenced in this project. Distances between genes are labeled in base pairs. n/a, not available due to gaps in sequence assembly.

Fig. 2.

Alignment of *Ae. aegypti*, *An. gambiae*, and *D. melanogaster* hsp70-predicted proteins. Gene sequences listed in the Materials and Methods section were aligned using MEGA 4.1 and bootstrap analysis was performed using the neighbor-joining method with 1,000 replications. Bootstrap support (>80) is listed on the node of each branch, where applicable. AaHsp70Bb' (repaired) indicates the predicted protein after removing two deleterious mutations.

Fig. 3.

Sequence comparison of *AaHsp70* genes. Complete transcripts for six *AaHsp70* genes were aligned using ClustalW (Geneious, Biomatters, Auckland, New Zealand) to produce an identity graph. The start of each open reading frame is marked with a green arrow, and the end with a red arrow. Dark marks on lines representing each *AaHsp70* gene indicate base changes from the consensus *AaHsp70* sequence.

Fig. 4.

Expression levels of *AaHsp70* genes increase with heat shock temperature. Female *Ae. aegypti* were heat shocked at temperatures from 35 to 39°C, and total RNA was hybridized to a ^{32}P -labeled probe common to all *AaHsp70* genes. All blots were hybridized simultaneously to ensure equivalent exposure and probe normalization (specific activity = 5.4×10^7 CPM). Control mosquitoes (cnt) were not heat shocked. Ethidium bromide-stained rRNA loading controls are shown below each blot.

Fig. 5.

Northern analysis of individual *AaHsp70* paralogs. Northern analysis was performed using a ^{32}P -labeled probe derived from the 3' untranslated regions of *AaHsp70* paralogs. Exposure times were standardized based on specific activity of each probe (7.3×10^7 – 3.0×10^8 CPM). Mosquitoes were heat shocked at 39°C , and control mosquitoes (cnt) were not heat shocked. Ethidium bromide–stained rRNA loading controls are shown below each blot.

Fig. 6.

Tissue and life stage expression of *AaHsp70* genes. Expression in specific tissues (A) and life stages (B) was analyzed by Northern analysis using a ^{32}P -labeled probe designed to hybridize to all *AaHsp70* genes. Tissues were dissections from female *Ae. aegypti* that had been heat shocked at 39°C for 1 h or kept at 28°C as controls (C). Tissues include whole body (WB), head (H), salivary glands (SG), thorax without salivary glands (T), ovaries (O), and midguts (M). RNA from males (σ), third and fourth instar larvae (L), and pupae (P) heat shocked at 39°C for 1 h was used for the life stage study. Ethidium bromide-stained rRNA loading controls are shown below each blot.

Table 1Oligonucleotide primers used to amplify and clone *AaHsp70* paralogs

Primers ^a	Sequence (5' to 3')	Expected length (bp)
Aa_F	ATCAAGATTGTCGACCAACTAGAAGGACC	2,398
Aa_R	TTTCTACTATATAAGCGCCCGGTTTCG	
Ab_F	TCGTTTCCACTATATAAGCGCCCAGC	2,346
Ab_R	CTGAGTTCGGTATGTTTGAGAATGAGAATG	
Ba_F	CGCGTCTTCCAACATTCCTTACTGAACCTA	2,374
Ba_R	CCACTCACACAAAGCAATGAAATATGCGAC	
Bb_F	CGAATCACAAGCAAGAGCAATAAAGCGCC	2,463
Bb_R	GGCAATTCGTAGCTCTATTGACATGTCCAG	
Ca_F	GGTAGCAGTCGAGTAAGCCAAGACAACGAA	2,334
Ca_R	AACAAGCTCAACATAATAGGAACATTAACAAACTTCC	
Cb_F	CCTGGCGTTTATATATAGGACCGATTGAGC	2,374
Cb_R	AAGCATTAGCTTGAGTCGTCAATCTTTAAGATTACTG	

^a Cloning primers contained the sequence *tttgggtacc* at the 5' end of each of the above oligonucleotides to introduce *KpnI* restriction sites.

Table 2
tBLASTn search with the *D. melanogaster* *Hsp70Aa* gene product yields 6–12 *Ae. aegypti* orthologs

Gene	Supercontig		Predicted protein			Accession no.
	No.	(Position)	No. AA	MW (kD)	Best Dm ^a	
<i>AaHsp70Aa</i>	1.680	(378,927–380,840)	638	70.3	<i>Hsp70Bb</i>	FJ177309
<i>AaHsp70Ab</i>	1.680	(383,454–385,367)	638	70.3	<i>Hsp70Bc</i>	FJ177310
<i>AaHsp70Ba</i>	1.680	(402,097–404,010)	638	70.3	<i>Hsp70Bc</i>	FJ177311
<i>AaHsp70Bb</i>	1.680	(406,760–408,673)	638	70.0	<i>Hsp70B</i>	FJ177312
<i>AaHsp70Ca</i>	1.680	(416,064–417,977)	637	70.0	<i>Hsp70B</i>	FJ177313
<i>AaHsp70Cb</i>	1.680	(419,055–420,962)	636	70.0	<i>Hsp70B</i>	FJ177314
<i>AaHsp70Aa'</i>	1.824	(45,244–47,157)	638	70.2	<i>Hsp70B</i>	n/a
<i>AaHsp70Ab'</i>	1.824	(49,384–51,297)	638	70.3	<i>Hsp70B</i>	n/a
<i>AaHsp70Ba'</i> ^b	1.824	(61,070–63,023)	n/a	n/a	n/a	n/a
<i>AaHsp70Bb'</i>	1.824	(66,155–68,064)	638 ^c	70.4	<i>Hsp70Bc</i>	n/a
<i>AaHsp70Ca'</i>	1.824	(86,660–88,569)	638	70.1	<i>Hsp70Bc</i>	n/a
<i>AaHsp70Cb'</i>	1.824	(89,650–91,384)	636	70.1	<i>Hsp70Bc/Bb</i>	n/a
<i>AaHsc70-2</i>	1.389	(987,893–989,673)	587	64.4	<i>Hsc70-2</i>	n/a
<i>AaHsc70-4</i>	1.28	(547,680–549,359)	593	65.2	<i>Hsc70-4</i>	n/a
<i>AaHsc70-4</i>	1.116	(1,035,564–1,037,399)	651	71.1	<i>Hsc70-4</i>	n/a
<i>AaHsp68</i>	1.752	(334,685–336,619)	624	68.8	<i>Hsp68</i>	n/a
<i>AaHsc-3a</i>	1.191	(572,103–574,019)	655	72.3	<i>Hsc70-3</i>	n/a
<i>AaHsc-3b</i>	1.191	(574,474–576,459)	662	73.4	<i>Hsc70-3</i>	n/a

^a Best match in *D. melanogaster* after BLASTp search of Flybase (FB2008_10).

^b Not available because of major gaps in the sequence assembly.

^c Two distinct mutations (nonsense and 1-bp deletion) interrupt the coding sequence of this gene; the data are presented as if the ORF were intact.

AA, amino acid; MW, molecular weight; n/a, not available.

Table 3
Nucleotide identity matrix of *AaHsp70* open reading frames on supercontig1.680

	Aa	Ab	Ba	Bb	Ca	Cb
<i>AaHsp70Aa</i>	1.000	0.937	0.935	0.910	0.914	0.774
<i>AaHsp70Ab</i>		1.000	0.935	0.899	0.904	0.775
<i>AaHsp70Ba</i>			1.000	0.908	0.909	0.783
<i>AaHsp70Bb</i>				1.000	0.956	0.782
<i>AaHsp70Ca</i>					1.000	0.781
<i>AaHsp70Cb</i>						1.000