The *Hsp27* gene is not required for *Drosophila* development but its activity is associated with starvation resistance

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Abstract Heat shock proteins are induced under stress conditions and they act as molecular chaperones to refold denatured polypeptides. Stress resistances including thermotolerance generally are correlated with levels of the heat shock proteins. We investigated a fruit fly gene encoding a small heat shock protein, *Hsp27*, to determine if it functions in stress response of *Drosophila melanogaster*. A knockout *Hsp27* allele was generated. Flies homozygous for this allele were viable, without obvious defects, and fertile, indicating *Hsp27* is not essential for development. In stress-response tests, loss of the *Hsp27* gene caused no defects in resistance to heat shock or oxidative treatments. However, a significant reduction in starvation resistance was associated with the genotype without a functional *Hsp27* gene. The data suggest that the *Drosophila* HSP27 protein acts as a chaperone to provide cellular stress resistance, although its function may be limited to a subset of the stress response such as the starvation resistance.

INTRODUCTION

Heat shock response is a cellular mechanism that is induced upon exposure to increase in temperature and it provides thermotolerance to the cells (Lindquist 1986; Morimoto 1993). In response to heat shock, a large group of ubiquitous, phylogenetically conserved proteins, known as heat shock proteins (HSPs), are induced in both prokaryotes and eukaryotes. HSPs are molecular chaperones that confer proper protein folding and play important roles in stress response by preventing the accumulation of damaged or misfolded proteins in cells (Soti and Csermely 2000; Hartl and Mayer-Hartl 2002). Studies also have shown general correlations between overexpressing HSPs and elevated cellular resistance to stress conditions, such as heat shock and oxidative stress. (Parsell et al 1993; Feder et al 1996; Morrow et al 2004b; Wang et al 2004).

In normal nonstressed prokaryotic cells, several HSPs act as molecular chaperones in assisting the folding process of newly synthesized proteins (Hartl and Hayer-

Hartl 2002). In multicellular organisms, the *hsp* gene expression is spatially and temporally regulated, suggesting that they play important roles under normal physiological conditions (Michaud et al 1997; Christians et al 2003; Krone et al 2003). Indeed, some of the HSPs are essential for development. For example, the *Hsp83* gene of *Drosophila* is required for signal transduction, and loss of its function results in developmental failure (Van Der Straten et al 1997; Rutherford and Lindquist 1998). The mitochondrial *Hsp60* gene of *Drosophila* is essential for fertility, and loss of its activity is detrimental to the fly development (Perezgasga et al 1999). Another gene closely related to the *Hsp60* gene, *Hsp60B*, is required exclusively for *Drosophila* late spermatogenesis (Timakov and Zhang 2001). In mice, the *Hsp70-2* gene also is required for spermatogenesis (Eddy 1999).

Small heat shock proteins ([sHSPs], ~15–40 kDa) initially were found in *Drosophila melanogaster* (Tissieres et al 1974), but their physiological functions largely remain unknown. These ubiquitous proteins share the α -crystallin domain of approximately 90 amino acid residues near their C-termini (de Jong et al 1998). The N-termini and the tails at the C-termini of different sHSPs vary extensively. A number of sHSPs form large oligomers in cells

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and the oligomers bind to unfolded proteins (Van Montfort et al 2001; Giese and Vierling 2002). Investigations into the mechanisms of unfolding and refolding proteins suggest that the primary function of sHSPs is to protect denatured proteins from aggregation by forming complexes with malfolded polypeptides (Jakob and Buchner 1994; Ehrnsperger et al 2000; Morrow et al 2006). sHSPs are implicated in a variety of cellular activities, including thermotolerance, resistance to apoptosis, and eye lens transparency (Landry et al 1989; Arrigo 1998; Liang and MacRae 1999; Andley et al 2002). In this study we took a genetic knockout approach to investigate the sHSP functions in *Drosophila melanogaster*. We report the isolation and characterization of a null allele of the *Hsp27* gene. The results show that the loss of the *Hsp27* function caused no obvious developmental defects, because flies homozygous for the null allele were viable, normal-looking, and fertile. However, the homozygotes displayed a reduced stress response to starvation and a decrease in mean life span.

MATERIALS AND METHODS

Drosophila stocks

All flies were grown on standard cornmeal/agar media at 25°C. A stock containing a P-element insertion, EP(3)3583, was described previously (Timakov et al 2002). An isogenetic strain containing the *w¹¹¹⁸* allele initially was obtained from the Bloomington *Drosophila* Stock Center, and a pair of flies was taken from the stock to establish a fresh isogenic strain for the studies described here.

Mutagenesis to generate a null allele of the *Hsp27* gene

To isolate a null allele of the *Hsp27* gene, we activated the EP(3)3583 insertion in the promoter region of the *Hsp27* gene by using a transposase source as described previously (Timakov et al 2002). We then monitored changes of the expression of a marker gene, the *mini-white* gene, that is carried within the EP(3)3583 element. The *mini-white* gene in P-element transformation vectors is derived from the X-linked *white* gene (Levis et al 1985; Pirrotta 1988). Regulatory elements necessary for high levels of *white* expression in the adult eyes are absent from the *mini-white* gene. Flies carrying a *mini-white* transgene displayed pale yellow to orange-red eyes, depending on the genomic sites where the transgene is inserted. We reasoned that the *mini-white* expression of the EP(3)3583 insertion could be modified as the flanking genomic sequence was changed through P-element-induced rearrangement (Zhang and Spradling 1993; Preston et al

1996). After activating the EP(3)3583 insertion by the transposase, flies displaying altered eye pigmentation were collected and individual stocks were established from these flies.

Polymerase chain reactions primers

The polymerase chain reaction (PCR) primers were

Phsp27-a:	5'-ACATTGGGTGTGTTGTGG-3'
Phsp27-b:	5'-GGATCATGACTACCGCAC-3'
Pg-R:	5'-GAGCCAGAAGATGCGAGA-3'
Pp31:	5'-CGACGGGACCACCTTATGTTATTT CATCATG-3' (the 31-bp inverted terminal repeat of the P element and with an outward orientation)
Pactin-a:	5'-CGCTCATTTCCGATGGTG-3'
Pactin-b:	5'-GGCTCCTTTGAACCCCAAG-3'
Hsp27RT-upl:	5'-GGTCGTCGTCGTTATTCG-3'
Hsp27RT-lowl:	5'-TTGAACTGCGACACATCC-3'
Rp49-fwd:	5'-TGTCCTTCCAGCTTCAAGATGACC ATC-3'
Rp49-ref:	5'-CTTGGGCTTGCGCCATTTGTG-3'

Reverse transcriptase–polymerase chain reaction and real-time PCR

Total RNA was isolated from thirty 2-d-old flies. For paraquat treatment, flies were fed with 5% sucrose containing 20 mM paraquat for 18 h. For starvation treatment, flies were kept in a plastic vial containing a wet filter paper for 24 h.

Total RNA was isolated by using an Absolutely RNA[™] reverse transcriptase–polymerase chain reaction (RT-PCR) Miniprep Kit (Stratagene Inc., La Jolla, CA, USA) following the manufacture's instructions. cDNA libraries for different stress treatments were made by using a GeneAmp[®] RNA PCR kit (Roche Inc., Indianapolis, IN, USA). For regular RT-PCR, polymerase chain reactions were carried out using GeneAmp system (PE Applied Biosystem Inc., Foster City, CA, USA) under standard conditions on a Robocycler (Stratagene Inc.). For real-time PCR, quantification of mRNA expression was performed using a MyiQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). SYBR Green (Bio-Rad Laboratories) was used as a double-stranded DNA-specific fluorescent dye. Each of the quantitative reactions was carried out in triplicates. To generate relative values of the *Hsp27* abundance in the cDNA libraries, the comparative cycle threshold method was used (Pfaffl 2001). Value of the *Hsp27* expression was determined by calculating the difference of comparative cycle thresholds between the *Hsp27* expression under each of the three conditions (non-stress treatment, oxidative treatment, or starvation treatment) and a calibrator gene, *Rp49*.

Sequencing

PCR product amplified from the genomic DNA was loaded on a gel of 0.7% low melting temperature agarose (FMC Bioproducts Inc., Philadelphia, PA, USA) in the TAE buffer, and the band containing the amplified DNA was cut out. The gel-purified DNA was used as a template for a second round of PCR with the same primers used in the first PCR reaction. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) and was used in a sequencing reaction (PE Applied Biosystem Inc.) with the same pair of primers.

Heat shock treatment

Two separate tests were used to examine the effect of heat shock treatments. In the first test, 3- to 4-d-old males were transferred into empty plastic vials containing filter papers saturated with 5% sucrose. Flies in the vials (20 per vial) were pretreated at 35°C by immersing the vials in a circulated water bath for 30 min and were allowed to recover for 1 h at 25°C. The flies then were transferred into a water bath at 37°C for various lengths and thermotolerance was measured as the percentage of flies displaying body movement 12 h after the heat shock treatment.

In the second test, vials with a thin layer of fresh medium were warmed to the experimental temperature at 35°C. Three- to four-day-old males were introduced into the vials and exposed to heat stress in a circulated hot-air chamber set at 35°C and 50–60% relative humidity. Mortality was examined every 4 h. A total of 100 flies in 5 groups were tested for each genotype. Mean survival time was calculated and subjected to nonparametric Wilcoxon signed-rank statistical test. Similarly, mean survivorship data from paraquat and starvation treatment were subjected to the same statistical test by using the SAS programs.

Paraquat treatment

Three-day-old males were starved in empty vials for 6 h before the oxidative treatment with paraquat. The flies then were transferred into vials containing filter papers saturated with 200 μ l of 5% sucrose, 20 mM paraquat solution (Sigma Chemical Co., St Louis, MO, USA). Mortality was recorded every 4 h. A total of 100 flies in 5 groups for each genotype were tested.

Starvation treatment

Three-day-old males were starved in empty vials with cotton plugs saturated with water. A total of 100 flies in

Fig 1. *Hsp27* transcription from the allele associated with the EP(3)3583 element. Reverse transcriptase–polymerase chain reaction (RT-PCR) amplified from a pair of primers derived from the *hsp27* transcript, Phsp27-a and Phsp27-b, are shown. Three cDNA dilutions were 1:6, 1:3, and 1:1. Optimized PCR conditions were allowed for 28 cycles to ensure DNA amplification within the exponential range. RNA was extracted from young adult flies without or with heat shock pretreatment for 1 h at 37°C and recovery for 30 min at 25°C. As positive control for cDNA synthesis, amplification of a *Drosophila* actin cDNA with a pair of primers, Pactin-a and Pactin-b, also was shown. Wild type control was no heat shock (Lane 1–3) and heat shock (Lanes 7–9). The allele associated with the EP(3)3583 element was no heat shock (Lanes 4–6) and heat shock (Lanes 10–12).

5 groups were tested for each genotype. Mortality was recorded every 4 h.

Life span assay

Newly hatched males and females were collected and transferred to fresh bottles. They were kept in the bottles for 3 d to allow mating. Male and female flies then were separated. The males were maintained at 25°C, transferred to vials with fresh food every 2–3 d, and dead flies were counted upon each transfer. A total of ~270 flies were examined for each genotype. The assay was repeated once and similar data were obtained.

RESULT

Isolation of a null *Hsp27* allele

The *Hsp27* gene is present in a single copy in *Drosophila melanogaster*, and it is located within the 67B region of Chromosome 3. A P-element insertion, EP(3)3583, was mapped to the promoter region of the *Hsp27* gene and its insertion site is 89 bp 5' to the *Hsp27* transcription initiation site (Timakov et al 2002). Thus, the ORF of the *hsp27* allele associated with the P-element insertion remains intact. Flies homozygous for the chromosome with the insertion were viable and fertile. Because P-elements insert preferentially into 5' ends of genes and generate mutant alleles that partially disrupt transcriptional activities of the affected genes (Spradling et al 1995), we asked if the allele associated with EP(3)3583 is transcriptionally active. By using RT-PCR with a pair of primers derived from the *Hsp27* transcript, we have determined that the *Hsp27* allele associated with EP(3)3583 was transcribed, though its mRNA level was lower than that of a wild type allele, indicating that the *Hsp27* allele is leaky (Fig 1).

To address the question of whether the *Hsp27* gene is

Fig 2. The *hsp27*^{-0.7} null allele. (A) A schematic drawing of the genomic DNA flanking the EP(3)3583 element. The relative positions of the *Hsp27* transcript and the deletion in the *hsp27*^{-0.7} allele are shown. The breakpoints of the deletion (707 basepair, from +179 to +885) are separated by an insertion of 3 nucleotides (GAA, underlined). (B) Amplification of genomic DNA by polymerase chain reaction (PCR). With the Pp31 and Phsp27-a primer pair a fragment of ~1.1 kb was produced from the starting chromosome with EP(3)3583 (control, Lane 1), whereas a fragment of ~0.4 kb was produced from the *hsp27*^{-0.7} allele (Lane 2). With the Pp31 and Pg-R primer pair a fragment of ~250 bp was produced from the starting chromosome with EP(3)3583 (control, Lane 3) and from the *hsp27*^{-0.7} allele (Lane 4).

essential for *Drosophila* development, we took a further step to generate a null allele for the *Hsp27* gene. As described in the *Materials and Methods*, we activated the EP(3)3583 element and established a total of 145 lines that displayed altered eye pigmentation and therefore were candidates for harboring a null *Hsp27* allele. We employed standard PCR to isolate a null allele among these lines. Genomic DNA was isolated from each of the candidate strains and used as a template for PCR with a pair of primers, Pp31 and Phsp27-a (Fig 2). The Pp31 primer is derived from the 31-bp terminal inverted repeats of the P-element, and the Phsp27-a primer is derived from the *Hsp27* gene. All but one of the strains produced a 1,078 bp PCR product that was generated between Phsp27-a and the starting EP(3)3583 element. The exceptional strain produced a ~0.4 kb PCR product, which was much smaller than that of the normal product of 1,078 bp with the Pp31 and Phsp27-a primer pair (Fig 2, lanes 1 and 2). The result from PCR experiments with another pair of primers, Pp31 and Pg-R, indicated that the 5' end of the

starting EP(3)3583 element was retained in this line, because the size of the PCR product from this line was the same as that of the starting line (241 bp, Fig 2, lanes 3 and 4). Further characterization by Southern blot analysis suggested that the starting element was retained, but a deletion was present between the Pp31 and Phsp27-a primers (data not shown). We determined the molecular structure of the deletion by sequencing the ~0.4 kb PCR product with the Pp31 and Phsp27-a primers. The data showed a 707 bp deletion within the *Hsp27* gene. The deletion removed the entire coding region of the *Hsp27* gene (213 aa). Sequencing data also revealed three additional nucleotides (GAA) at the junction of the deletion breakpoints (Fig 2). This *Hsp27* null allele was designated as *hsp27*^{-0.7}.

The *Hsp27* null allele caused no obvious developmental defects

Two steps were taken to introduce the *hsp27*^{-0.7} null allele into a near isogenic background. First, genetic crosses with balancers were used to introduce an isogenic *Chromosome X* and an isogenic *Chromosome 2* from an isogenic *w*¹¹¹⁸ strain (see *Materials and Methods* section) into the strain carrying the *hsp27*^{-0.7} allele. Second, females carrying the *hsp27*^{-0.7} allele in the isogenic background for *Chromosome X* and *Chromosome 2* were allowed to backcross to males of the isogenic *w*¹¹¹⁸ strain for 10 generations. During this introgression process, the *hsp27*^{-0.7} allele was recovered by recognizing its *mini-white* marker gene expression from the retained EP element in *hsp27*^{-0.7}. After 10 generations with meiotic recombination, a single fly carrying the *hsp27*^{-0.7} allele, which was confirmed by PCR (Fig 2), was used to establish a fresh stock in a near isogenic background.

Homozygotes for the *hsp27*^{-0.7} allele in the near isogenic background were healthy with no obvious visible defects. Fertility tests also showed that both of homozygous males and females were fertile, at levels comparable to wild type controls. The numbers of eggs produced from the homozygotes, heterozygotes, and *w*¹¹¹⁸ wild type controls were monitored and the rates of the egg productions were determined (Table 1). The homozygotes produced 179.4 ± 11.2 (Mean $\pm$ standard deviation [SD]) eggs per female from day 3 to day 26 after eclosion. Though it was slightly more than that of the heterozygotes (166.4 ± 10.0), or the wild type control (155.6 ± 20.1), the difference is statistically insignificant ($P = 0.09$ in both cases, Table 1). The rates of egg production were similar among the homozygotes, heterozygotes, and wild type controls for the young females (4- to 7 d-old) or the older females (21- to 24 d-old; Table 1). These data indicate that the null allele did not affect the egg production. Furthermore, from a cross between $+/\textit{hsp27}^{-0.7}$ siblings, the progeny

Table 1 Total number of eggs and the egg production rate

Genotypes	Total ^a		Young ^b		Older ^c	
	Eggs/female (mean ± SD)	P-value	Eggs/female (mean ± SD)	P-value	Eggs/female (mean ± SD)	P-value
<i>hsp27^{-0.7}/hsp27^{-0.7}</i>	179.4 ± 11.2		41.8 ± 2.8		20.9 ± 2.7	
<i>hsp27^{-0.7}/+</i>	166.4 ± 10.0	0.09	36.6 ± 5.0	0.11	23.5 ± 4.6	0.55
<i>+/+</i>	155.6 ± 20.1	0.09	36.4 ± 2.8	0.25	22.9 ± 6.0	0.13

SD, standard deviation.

The test was carried out in vials with five females and five males per vial. A set of 5 vials was used for each genotype.

^a Total numbers of eggs per female produced from day 3 to day 26 after eclosion.^b Number of eggs per female produced from 4- to 7-d-old females.^c Number of eggs per female produced from 21- to 24-d-old females.

showed a ratio of 1:2:1 among the *+/+*, *+/hsp27^{-0.7}*, and *hsp27^{-0.7}/hsp27^{-0.7}* genotypes (952, 2065, 945, or very close to the 1:2:1 ratio). Taken together, these results show that the *Hsp27* gene is not required for fly development under the laboratory conditions.

Heat and oxidative stress resistance

To study the roles of the *Hsp27* gene in stress response, 3 series of experiments were carried out to examine the effect of the *hsp27^{-0.7}* null allele on stress resistance under heat shock stress, oxidative stress, or starvation conditions. Young flies of the same age (3- to 4-d-old adults) were used in all experiments. In addition, each test on the effect of the stress resistance was repeated at least twice.

Thermotolerance was shown to increase in severe heat shock treatment, if a mild heat shock, known as pretreatment, was administrated (Mitchell et al 1979). To examine the effect of the loss of the *Hsp27* gene activity on thermotolerance, we constructed 3 genotypes, the *hsp27^{-0.7}/hsp27^{-0.7}* homozygote, its heterozygous sibling, and a wild type control. After a pretreatment, a recovery peri-

od, and severe heat shock treatment (see *Materials and Method* section), the differences of the survivorships among the genotypes were small and statistically insignificant (Fig 3A). The thermotolerance tests with pretreatment indicate that loss of the *Hsp27* activity does not cause a significant reduction in heat shock resistance.

High levels of the HSP70 protein generally are correlated with increased thermotolerance in *Drosophila* (Parsell et al 1993; Feder et al 1996). In addition, elevated thermotolerance also was seen when sHSPs were overexpressed (Morrow et al 2004b; Wang et al 2004). We asked if stress-induced high levels of *Hsp27* also could increase thermotolerance. In *Drosophila* cell culture, heat shock induces the expression of sHSP genes, including *Hsp27* with an optimal temperature of approximately 35°C (Yost and Lindquist 1986). To examine the effect of the *Hsp27* gene on thermotolerance under conditions where its induction is optimal, genotypes with or without the *Hsp27* gene were treated continuously at 35°C. The rate of adult death under the heat shock condition was determined for each genotype. When exposed to 35°C, the *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes displayed a mean survivorship of 31.9 ± 1.0 h, although its heterozygous siblings

Fig 3. Effect of the *hsp27^{-0.7}* allele on thermotolerance. (A) Thermotolerance at 37°C with a mild pretreatment. Survivals (%) of 3 genotypes are shown. (B) Thermotolerance under conditions of optimal sHSP induction (35°C). Mean survivals (hours) of 3 genotypes are shown. Genotypes: *hsp27^{-0.7}/hsp27^{-0.7}*, *hsp27^{-0.7}/+*, and *+/+*. The *w¹¹¹⁸* strain was used as a wild type control. Values are means with 95% confidence intervals.

Fig 4. Effect of the *hsp27^{-0.7}* allele on paraquat treatment. Mean survivals (hours) of 3 genotypes are shown: *hsp27^{-0.7}/hsp27^{-0.7}*, *hsp27^{-0.7}/+*, and *+/+*. The *w¹¹¹⁸* strain was used as a wild type control. The 20 mM paraquat was in 5% sucrose solution. Values are means with 95% confidence intervals.

and wild type controls showed 30.4 ± 1.5 h and 31.3 ± 1.5 h of mean survivorship, respectively (Fig 3B). Thus, loss of the *Hsp27* function appeared to have no adverse effect on the heat-induced death rate at 35°C. In conjunction with the thermotolerance data at 37°C obtained from the tests with pretreatment, these results suggest that *Hsp27* is not required in heat shock resistance.

We also examined the effect of the *hsp27^{-0.7}* null allele on oxidative stress resistance by exposing flies to paraquat, an oxidative reagent. The *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes showed oxidative resistance at levels comparable to that of wild type controls (a mean survivorship of 63.5 ± 13.0 h for *Hsp27* null homozygotes vs. 65.2 ± 8.6 h for a wild type control, Fig 4). The oxidative resistance levels of the *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes were also similar to that of the *hsp27^{-0.7}* heterozygotes (mean survivorship, 72.1 ± 8.3 h, Fig 4). The data indicate that loss of the *Hsp27* function had no significant effect on the oxidative resistance.

Effect of the *Hsp27* null allele on starvation

The effect of the *hsp27^{-0.7}* null allele on starvation resistance also was measured in the rate of survivorship. The mean survivorship of a wild type control under starvation was 53.1 ± 2.7 h (Fig 5). In contrast, the mean survivorship of the *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes under the starvation test was only 32.6 ± 3.0 h, which was a decrease of 39% ($P < 0.01$). Similarly, the starvation-resistance levels of the *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes were significantly lower than those of the heterozygotes for the null allele (a mean survivorship of 47.9 ± 1.6 h, $P < 0.01$, Fig 5). The reduced resistance was seen for the null homozygotes shortly after administering the starvation treatment. Approximately 20 h into the starvation, the homozygotes displayed weakness in resisting the stress and began to die (Fig 6). At 32 h into the treatment, >40% of the homozygotes were dead, and its death rate was ~11

Fig 5. Effect of the *hsp27^{-0.7}* allele on starvation treatment. Mean survivals (hours) of 3 genotypes are shown: *hsp27^{-0.7}/hsp27^{-0.7}*, *hsp27^{-0.7}/+*, and *+/+*. The *w¹¹¹⁸* strain was used as a wild type control. Values are means with 95% confidence intervals.

Fig 6. The differential death rates on starvation. The rate of death was determined for two genotypes, *hsp27^{-0.7}/hsp27^{-0.7}* and a wild type control (*w¹¹¹⁸*), at various time periods (hours after the beginning of starvation). Values are means with 95% confidence intervals.

times higher than that of the control. These data suggest that the *Hsp27* gene plays a significant role in starvation resistance.

Loss of the *Hsp27* gene activity and life span

In addition to increase stress resistance, overexpressing several sHSPs in *Drosophila* extended the life span (Walker and Lithgow 2003; Morrow et al 2004b; Wang et al 2004). To address a question of whether loss of the *Hsp27* gene influences the aging process, we examined the male life spans of three genotypes, *hsp27^{-0.7}/hsp27^{-0.7}* homozygote, *hsp27^{-0.7}/+* heterozygote, and a wild type control at 25°C. Compared with the wild type or the heterozygous controls, there was an early acceleration of death for the *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes at the age of approximately 35 d old (Fig 7). Although the wild type controls and the *hsp27^{-0.7}/+* heterozygotes had mean life spans of 56.4 d and 61.3 d, respectively, the *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes only had a mean life span of 45.2 d. Thus, male *hsp27^{-0.7}/hsp27^{-0.7}* homozygotes showed a 19.8% ($P < 0.001$) decrease in the mean life span when compared to that of the wild type control, or a 26.2% ($P < 0.001$) decrease of the mean life span when compared to the *hsp27^{-0.7}/+* heterozygote. In addition, the reduction in the

Fig 7. Effect of the *hsp27*^{-0.7} allele on life span. Flies were maintained in a humidified and temperature-controlled environmental chamber (25°C, 50–60% relative humidity). A total of 279 *hsp27*^{-0.7}/*hsp27*^{-0.7} homozygous males and 268 *w*¹¹¹⁸ control males were tested. Two-sample T-test showed a significant difference of lifespan between the homozygotes and the control ($P < 0.001$).

Fig 8. *Hsp27* transcription under starvation and oxidative conditions. cDNA libraries were constructed from wild type adult flies. Levels of transcription for the *Hsp27* gene were determined using real-time polymerase chain reaction (PCR), under stress conditions of oxidative treatment with paraquat, and starvation treatment. The *Hsp27* transcription level of a nonstress control is set at 1.0 (100%). Values are means with 95% confidence intervals. Pairs of primers for the amplification of the *Hsp27* transcript were *Hsp27*RT-up1 and *Hsp27*RT-low1. The expression level of the calibrator gene, *Rp49*, in each of the cDNA libraries was determined with a pair of primers *Rp49*-fwd and *Rp49*-rev.

hsp27^{-0.7}/*hsp27*^{-0.7} life span was correlated with an increase of deaths that began at ~30 d of age, although the maximum life span appeared to be unaffected.

Inductions of the *Hsp27* transcription under oxidative or starvation treatment

The influence of either an oxidative treatment with paraquat, or starvation treatment, on the transcription of the *Hsp27* gene was examined by using real-time PCR. The quantitative analysis shows that the abundance of the *Hsp27* transcript is not affected significantly by either of the stress treatments, when total mRNA isolated from the adults is examined (Fig 8). When compared to a non-stressed control, the related abundance of the *Hsp27* transcript for the paraquat-treated sample is 1.02 ± 0.01 , indicating no change of the *Hsp27* transcript level between the control and the oxidative treated sample. For the starvation-treated sample, the related abundance of the *Hsp27*

transcript is 0.91 ± 0.02 . Though it appears to be slightly lower than the control (1.00 ± 0.12), the difference is not significant ($P = 0.24$).

DISCUSSION

The spatially and temporally regulated expression patterns of the sHSPs and the proposed sHSP chaperone activities suggest that sHSPs carry out important physiological functions as chaperones under normal and non-stressed conditions. During the *Drosophila* larval development, *Hsp27* is expressed primarily in the brain and gonads (Pauli et al 1990; Michaud et al 1997). In the adult tissues it is localized in the *Drosophila* central nervous system and the germ line cells of both males and females. The present studies showed that loss of the *Hsp27* function caused no obvious developmental defects in *Drosophila*. Flies homozygous for the loss-of-function allele were viable and fertile. Thus, our results demonstrated that the *Drosophila* *Hsp27* gene is required for neither viability nor fertility. Moreover, because the null allele causes no obvious phenotypes, we conclude that the *Hsp27* gene does not play significant roles in *Drosophila* development under the laboratory conditions. Similar results were obtained for a sHSP gene in *S. cerevisiae*, *Hsp26*, showing that it is not required for physiological functions (Susek and Lindquist 1989). In *Drosophila melanogaster*, there are four well-characterized sHSP genes, including *Hsp22*, *Hsp23*, *Hsp26*, and *Hsp27*. It is possible that they overlap functionally during development. Thus removing *Hsp27* from the gene family may cause no detectable developmental defects. In addition to the sHsp genes in *Drosophila*, a database search revealed that there are at least 6 additional genes related to sHSP genes because each carries a recognizable α -crystallin domain. They are distributed on various chromosome locations, with one on the X, another on Chromosome 2, and 3 more on Chromosome 3.

The involvement of sHSPs in the cellular stress-response mechanisms has been implicated in many studies. Mutation analyses of the bacterial sHSP genes revealed essential functions of several sHSP chaperones in stress resistance (Servant and Mazordier 1995; Kitagawa et al 2000; Lee et al 2000). A growing body of evidence also indicates that sHSP are involved in a variety of cellular stress-response activities in multicellular organisms. Studies in *Drosophila* showed that upregulating the sHSP expression was associated with elevated resistance to a number of stress treatments. Flies that carried constructs of overexpressing *Hsp26*, or *Hsp27*, displayed increased stress resistance against oxidative, heat, or starvation treatments (Wang et al 2004). Although high levels of *Hsp22* expression in adults might be toxic (Bhole et al 2004), there was also a correlation between levels of the

Hsp22 expression and stress resistance (Morrow et al 2004a, 2004b).

Our results showed that loss of the *Hsp27* function was associated with a significant decrease of stress response to starvation. However, loss of the *Hsp27* function did not show a significant effect on the stress resistance to heat shock, or to paraquat oxidative treatment, under the test conditions. Thus, overexpressing *Hsp27* influenced *Drosophila* resistance to heat shock and oxidative stress conditions, but loss of the *Hsp27* function had no significant effect on these stress conditions. These different stress responses between the *Hsp27* overexpression and the loss of the *Hsp27* function could be due to an ectopic nature of gene induction associated with the overexpression system, which activates the *Hsp27* transcription ubiquitously in the adult cells (Wang et al 2004). The ectopic *Hsp27* expression also might induce a gain-of-function mechanism of stress response in some tissues that normally do not express *Hsp27*. Because loss of the *Hsp27* function was associated with a defect in starvation resistance, but not in heat or oxidative resistance, our data suggest that individual sHSPs perform distinct cellular functions in response to various stress conditions.

Although the stress tests reveal a significant influence of the *Hsp27* gene activity on starvation, our quantitative RT-PCR analyses show no significant change of the *Hsp27* transcript level under conditions where the adult flies were treated with starvation. It is possible that the effect of the *Hsp27* gene on starvation resistance does not require a transcriptional response on the *Hsp27* gene. Several phosphorylated isoforms of the HSP27 protein were reported previously (Marin et al 1996). Posttranscriptional modifications such as phosphorylation to the HSP27 protein, which could be downstream of a primary stress-response mechanism, might modulate the HSP27 protein, giving rise to the effect of the *Hsp27* gene on starvation. To reconcile the difference between the results from the stress tests and quantitative RT-PCR, it is also conceivable that, in response to a starvation treatment, an induction of the *Hsp27* transcription is restricted in a particular tissue, but the tissue-specific induction accounts for only a small fraction of the *Hsp27* transcript in the whole adult body. Thus, the real-time RT-PCR analyses, which were carried out using total adult cDNA libraries, might be unable to detect the small tissue specific induction.

Overexpressing a number of the sHSPs, including *Hsp16* in *Caenorhabditis elegans* and *Hsp22*, *Hsp26*, and *Hsp27* in *Drosophila*, extended the life span, in addition to the increase in stress resistance (Walker and Lithgow 2003; Morrow et al 2004b; Wang et al 2004). Knock-down mutations of the sHSP genes also were shown to be associated with shortened life span in *C. elegans* (Lin et al 2001; Hsu et al 2003). Thus, genetic manipulations have shown a direct correlation between level of the sHSP ex-

pression and the life span. Consistent with this correlation, our data showed that loss of the *Hsp27* function was associated with a decrease in stress resistance and life span. The sHSP genes are targets of a transcription factor, FOXO, a key member of the insulinlike signaling pathway that influences aging (Hsu et al 2003; Hwangbo et al 2004). Studies have further indicated that activation of the pathway in the brain is sufficient to control the life span in *Drosophila* and *C. elegans* (Wolkow et al 2000; Hwangbo et al 2004). Because it is localized in the nervous system of both larvae and adults, the *Drosophila Hsp27* may act as a downstream factor of the pathway in the brain to influence aging.

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