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Multiple functions of *Drosophila* heat shock transcription factor *in vivo*

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Heat shock transcription factor (HSF) is a transcriptional activator of heat shock protein (*hsp*) genes in eukaryotes. In order to elucidate the physiological functions of HSF in *Drosophila*, we have isolated lethal mutations in the *hsf* gene. Using a conditional allele, we show that HSF has an essential role in the ability of the organism to survive extreme heat stress. In contrast to previous results obtained with yeast HSF, the *Drosophila* protein is dispensable for general cell growth or viability. However, it is required under normal growth conditions for oogenesis and early larval development. These two developmental functions of *Drosophila* HSF are genetically separable and appear not to be mediated through the induction of HSPs, implicating a novel action of HSF that may be unrelated to its characteristic function as a stress-responsive transcriptional activator.

Keywords: development/heat shock/mutant/thermotolerance/transcription

Introduction

The heat shock response was identified originally in *Drosophila* as a change in the condensation of larval salivary gland polytene chromosomes elicited by temperature elevation (Ritossa, 1962), reflecting the activation of specific genes. This induction of gene activity appears to be a universal reaction of living cells to a variety of cellular stresses, elaborated by organisms as distantly related as bacteria and humans (reviewed in Lindquist, 1986). The induced genes encode a highly evolutionarily conserved class of proteins known as the heat shock proteins (HSPs); in *Drosophila*, this protein class is comprised of HSP83, HSP70 and the small HSPs (HSP22, 23, 26 and 28; reviewed in Lindquist, 1986; Lindquist and Craig, 1988). The synthesis of HSPs can be induced by a variety of conditions. Experimentally, common inducers are noxious stimuli, such as heat and chemicals, but cell injury, such as that caused by ischemia and infection, and in some instances cell proliferation and differentiation, can act as inducers as well (reviewed in Lindquist, 1986; Morimoto *et al.*, 1992). The function under stress of one of the HSPs has been clearly established: HSP104 of yeast has been shown to facilitate the resolubilization of protein aggregates under heat stress (Parsell *et al.*, 1994). The role of the other HSPs during cell stress is believed to be related to the essential functions performed by the constitutively expressed HSP cognates (HSCs), which modulate protein folding in the cell under non-stress conditions (reviewed in Lindquist, 1986; Welch, 1993). Presumably through their action as molecular chaperones, the HSPs contribute to thermotolerance, the ability of cells to survive a severe heat stress (reviewed in Parsell *et al.*, 1993).

The induction of synthesis of HSPs is regulated at both the transcriptional and translational levels (Lindquist, 1986). Central to transcriptional regulation in eukaryotes is the activity of the stress-regulated heat shock transcription factor (HSF; reviewed in Wu, 1995). HSF is present in unstressed cells in an inactive state and becomes activated in response to stress. In *Drosophila* and mammals, inactive HSF is monomeric and becomes converted to a trimer in response to heat stress, possibly through a switch from intramolecular to intermolecular coiled-coil interactions (Westwood *et al.*, 1991; Rabindran *et al.*, 1993; Zuo *et al.*, 1994). The trimeric form of HSF binds with high affinity to the heat shock element (HSE) that is present in *hsp* gene promoters and is composed of three or more inverted repeats of the consensus sequence nGAAn (Pelham, 1982; Amin *et al.*, 1988; Xiao and Lis, 1989). Once bound to DNA, HSF activates transcription through a potent transactivation domain in the C-terminus, which is negatively regulated in the absence of stress (Green *et al.*, 1995; Shi *et al.*, 1995; Zuo *et al.*, 1995; Newton *et al.*, 1996; Wisniewski *et al.*, 1996). In yeast, where HSF trimerization and hence DNA binding are generally constitutive, HSF activity is regulated primarily by an unmasking of the transactivation domain in response to heat stress (Nieto-Sotelo *et al.*, 1990; Sorger, 1990; Jakobsen and Pelham, 1991; Bonner *et al.*, 1992; Chen *et al.*, 1993).

The HSF of the yeast *Saccharomyces cerevisiae* has been shown to be required *in vivo* for the induction of HSP synthesis in response to heat stress (Smith and Yaffe, 1991). Interestingly, yeast HSF is also essential for cell growth or viability in the absence of stress (Sorger and Pelham, 1988; Wiederrecht *et al.*, 1988; Gallo *et al.*, 1993). This essential requirement may involve a role in the regulation of basal *hsp* gene expression (reviewed in Sorger, 1991). Several eukaryotic species studied have multiple HSFs, only one of which, HSF1, appears to be involved in the heat shock response (Wu, 1995). In mouse, a non-stress-regulated HSF, HSF2, has been proposed to play a role in normal developmental processes, as it becomes activated during erythrocyte differentiation and pre-implantation embryonic development (Sistonen *et al.*,

1992; Mezger *et al.*, 1994a,b). In order to elucidate the *in vivo* functions of HSF in a complex eukaryote, we have isolated four mutant alleles of the *Drosophila hsf* gene, including a null and a temperature-sensitive allele. The mutant phenotypes confirm the biochemical function of HSF *in vivo* and reveal novel roles for HSF in the normal development of *Drosophila*.

Results

Isolation of hsf mutations

The single-copy *Drosophila hsf* gene is located between the genes *staufen* (*stau*) and *Polycomblike* (*Pcl*) at cytological position 55A (Clos *et al.*, 1990; Lonie *et al.*, 1994). A *Pcl* mutation, Pcl^{P2}, was isolated by mobilizing a P-element inserted in *stau* (Lonie *et al.*, 1994). Using PCR and *in situ* hybridization analysis, we determined that the transposition event that generated the Pcl^{P2} mutation also deletes *hsf*. When DNA from individual embryos from the cross $Pcl^{P2}/+ \times Pcl^{P2}/+$ was subjected to PCR amplification, roughly one quarter of embryos tested, the frequency expected for Pcl^{P2} homozygotes, failed to show the presence of the *hsf* gene; a control amplification of a region of the *RpII140* locus, located elsewhere, gave a PCR product in the same reaction for all embryos (Figure 1B). To confirm this result, we analyzed polytene chromosome squashes from $Pcl^{P2}/+$ larvae by hybridization *in situ* with *hsf* cDNA. Only one of the two second chromosome homologs showed hybridization to the *hsf* probe at 55A, indicating that *hsf* is deleted in Pcl^{P2} (Figure 1C). By extending the PCR analysis to genomic regions neighboring *hsf*, we localized the proximal endpoint of the deletion in the Pcl^{P2} chromosome to the 5′ half of the *stau* transcription unit and the distal endpoint to the 5′ untranslated region of *Pcl* (Figure 1A, and data not shown).

In order to isolate mutations in *hsf*, we carried out two ethyl methanesulfonate (EMS) F_2 lethal genetic screens in *trans* to the Pcl^{P2} chromosome. The screening was based on the assumption that *hsf* is an essential gene in the absence of stress, as it is in yeast (Sorger and Pelham, 1988; Wiederrecht *et al.*, 1988; Gallo *et al.*, 1993). The results are summarized in Table I. From a total of 4989 chromosomes screened, three *Pcl* alleles were isolated. No *stau* alleles were isolated since *stau* mutations have a maternal-effect phenotype that would only be detected in the F_3 generation (Schupbach and Wieschaus, 1986). Additionally, four mutations were isolated that fell into a new complementation group. Genomic sequence analysis of the *hsf* gene in these four mutant lines identified a single base change in the *hsf* coding sequence of each (see below). Further, the mutations were rescued to viability, both when homozygous and in *trans* to Pcl^{P2}, by the construct P[w^+, hsf^+] containing the coding and upstream sequences of the *hsf* gene (Figure 1A). We conclude that this complementation group corresponds to *hsf*.

All four *hsf* mutations cause arrest at the 1st or 2nd larval instar stage of development. hsf^1 and hsf^3 behave as amorphic mutations, lethal when homozygous and in *trans* to the *hsf* deletion Pcl^{P2}. hsf^2 is a hypomorphic allele, giving rise to a small number of viable adults when homozygous but not in *trans* to Pcl^{P2}. hsf^4 is a temperature-sensitive mutation, viable at temperatures of 25°C and below, but not at 29°C (see Table I).

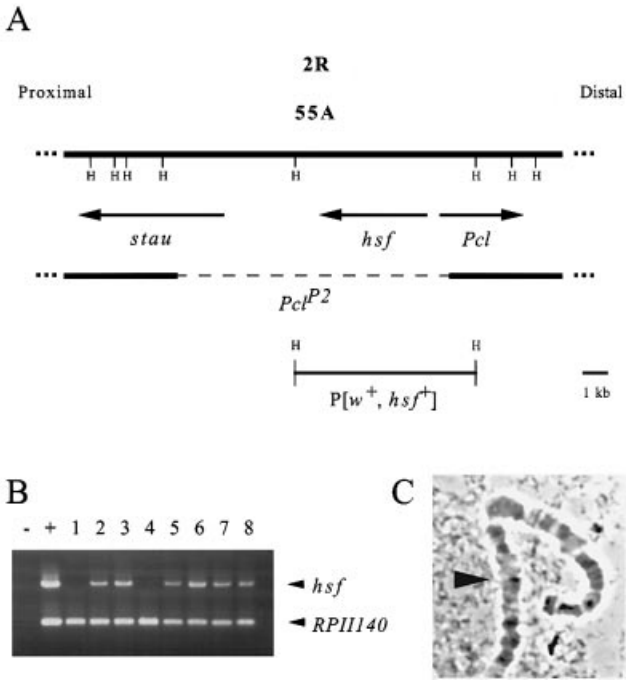

Fig. 1. Characterization of the Pcl^{P2} mutation that deletes *hsf*. (**A**) Genomic organization at 55A. The solid line at the top represents the wild-type chromosome (adapted from Lonie *et al.*, 1994 and R.Saint, personal communication; H: *Hin*dIII). The arrows below represent the transcripts from the region. The dashed line represents the extent of the deletion in the Pcl^{P2} mutant. The DNA contained in the rescue construct P[w^+, hsf^+] is also indicated. (**B**) PCR analysis shows that *hsf* is deleted in Pcl^{P2}. DNA from individual embryos derived from the cross $Pcl^{P2}/+ \times Pcl^{P2}/+$ was subjected to PCR amplification using primers to the *hsf* and *RpII140* (control) genes. Lanes – and + are products from reactions with and without wild-type *Drosophila* DNA, respectively; lanes 1–8 are reaction products from single embryos. (**C**) Polytene chromosomes from $Pcl^{P2}/+$ larvae were hybridized *in situ* with *hsf* cDNA probe. The right arm of chromosome 2 is shown with the hybridization signal corresponding to *hsf* (arrowhead) visible only on one chromosome homolog.

Table I. Summary of EMS F_2 lethal screens for *hsf* mutations

	Screen temperature	
	25°C	29°C[a]
Chromosomes screened in *trans* to Pcl^{P2}	1851	3138
hsf alleles isolated	2 (hsf^1, hsf^2)	2 (hsf^3, hsf^4)
Amorphic	hsf^1	hsf^3
Hypomorphic	hsf^2	
Null	hsf^1	
Temperature-sensitive		hsf^4

[a]This temperature was used in order to screen for temperature-sensitive mutations.

The sequence analysis of the *hsf* alleles is summarized in Figure 2. hsf^1 and hsf^2 are nonsense mutations at residues 78 and 373 respectively. The predicted hsf^1 product lacks all functional HSF domains and may be unstable *in vivo* since it could not be detected by Western blot of extracts from hsf^1 heterozygotes. Based on these data and the genetic analysis, we conclude that hsf^1 is a null allele. The predicted hsf^2 product was also undetectable by Western blot despite the presence of over half of the HSF

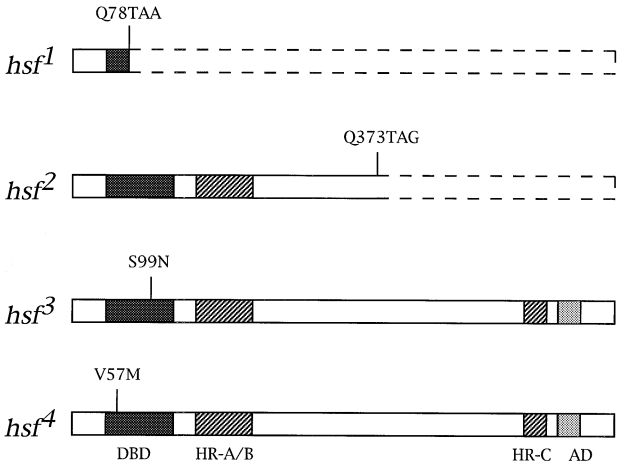

Fig. 2. Identity of *hsf* mutations. The organization of the functional domains of *Drosophila* HSF protein is shown. The DNA-binding domain (DBD), trimerization domain containing hydrophobic heptad repeats (HR-A/B), a domain also containing a hydrophobic heptad repeat and required for suppression of trimerization (HR-C) and the transactivation domain (AD) are indicated. The single nucleotide changes found in the *hsf* coding region of each of the four EMS-induced *hsf* mutants are shown as changes in the corresponding amino acid sequence. Amino acids are identified by the single-letter code; TAA and TAG are termination codons.

sequence, suggesting that it too is unstable *in vivo*. The occasional viability of hsf^2 homozygotes may be a consequence of translational read-through of the stop codon, as previously reported for yeast HSF (Kopczynski *et al.*, 1992). The hsf^3 mutation, S99N, is a non-conservative substitution at a serine residue in helix 3 of the DNA-binding domain that is invariant in all known HSFs and has been shown to be critical for DNA binding (Hubl *et al.*, 1994; Vuister *et al.*, 1994; S.J.Kim and C.Wu, unpublished observations). Since the hsf^3 protein product is detectable by immunostaining of cell clones homozygous for the mutation (unpublished observations), the hsf^3 mutation is likely to affect, at a minimum, the DNA-binding activity of HSF *in vivo*. The hsf^4 mutation, V57M, is a substitution at a highly conserved valine residue at the C-terminal end of helix 1 in the hydrophobic core of the DNA-binding domain (Vuister *et al.*, 1994). The effect of this temperature-sensitive mutation on HSF function may occur through a perturbation of the overall structure of the DNA-binding domain.

No heat shock response in the hsf^4 mutant

In order to investigate the physiological role of *Drosophila* HSF in the heat shock response, we tested the ability of the temperature-sensitive mutant hsf^4 to induce HSP synthesis. Since the temperature-sensitive period of the hsf^4 mutation is limited to early larval development (see later), viable late larvae and adults can be obtained for analysis when early development occurs at the permissive temperature. When homozygous hsf^4 adults were subjected to heat stress at 30, 33 and 36°C, no induction of HSP70 was observed at any of these temperatures by RNA dot-blot and Western blot analyses, in contrast to the expression in hsf^+ flies (Figure 3C and D). Induction of the other HSPs also was not observed, as determined by labeling of larval salivary glands *in vitro* with [^{35}S]methionine (data not shown). The inability to activate expression of

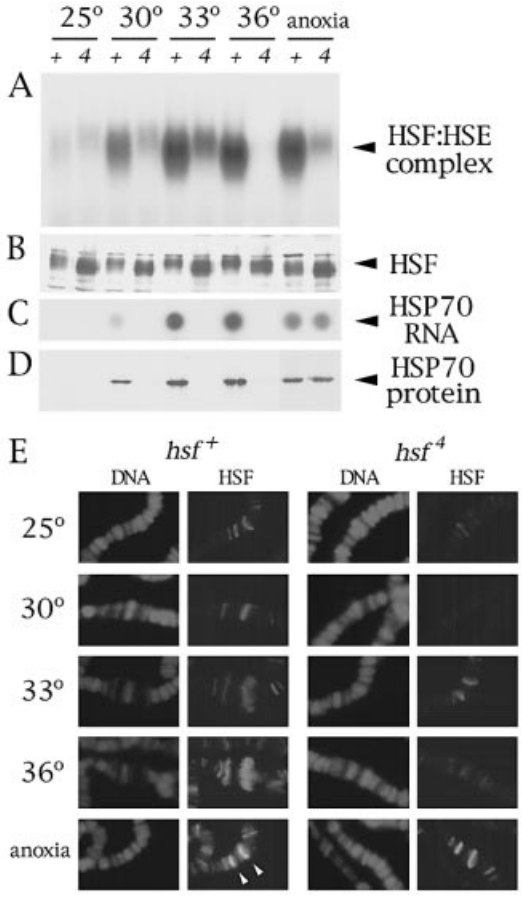

Fig. 3. Stress response in hsf^4 animals. (**A**) hsf^+ (+) and hsf^4 (4) adult flies were subjected to heat or anoxic stress. Heat stress was induced by incubation for 20 min at the indicated temperature. Anoxic stress was induced by administration of N_2 for 45 min, followed by a 45 min recovery in air during which HSP70 synthesis is observed (Lewis *et al.*, 1975). Protein extracts were prepared and were assayed for DNA-binding activity by EMSA with radiolabeled HSE oligonucleotide probe. The relative DNA-binding levels were reproducible between experiments despite small variations in the extraction of HSF from nuclei as judged by Western blot. In the anoxia experiments, HSF DNA binding became activated during the anoxic treatment (shown) and decayed during the recovery period. Similar results were obtained with late third instar larvae, both for heat shock and for anoxia using either N_2 or CO_2. (**B**) HSF Western blot of protein extracts from flies used in (A). The HSF^4 protein exhibited slighly higher electrophoretic mobility than wild-type for all treatments; we do not understand this difference, which may be due to differences in post-translational modification. (**C**) HSP70 dot-blot hybridization of RNA prepared from flies treated as in (A). (**D**) HSP70 Western blot of protein extracts from flies treated as in (A). (**E**) HSF localization to *hsp70* loci *in vivo*. hsf^+ and hsf^4 late third instar larvae were subjected to heat stress as in (A), or to anoxic stress by exposure to CO_2 for 30 min. DNA and HSF were visualized by propidium iodide staining and immunostaining with fluorescein-conjugated secondary antibody, respectively. The cytological positions of the *hsp70* loci are indicated in the bottom panel (left arrowhead: 87A, right arrowhead: 87C). The weak HSF localization observed in hsf^+ and hsf^4 samples at 25°C is probably due to stresses induced by tissue manipulation prior to fixation. In the anoxia experiments, the apparent DNA-binding activity of the HSF^4 protein measured by EMSA was noticeably lower than wild-type, even though the mutant exhibited near wild-type levels of both *in vivo* DNA binding, as measured by immunolocalization, and HSP70 induction. This discrepancy may be related to cooperative interactions between HSF^4 trimers binding to adjacent HSEs on the chromosomal *hsp70* promoter that are disallowed on the single HSE employed for EMSA. Alternatively, the anoxia-activated HSF^4 protein may be altered during extract preparation, leading to loss of DNA-binding activity *in vitro*.

hsp genes may be attributed to the attenuation (at 30 and 33°C) or the complete loss (at 36°C) of DNA-binding activity, as determined both by an electrophoretic mobility shift assay (EMSA) of *hsf*^{4} adult extracts and by immunostaining of larval polytene chromosomes for HSF localization to the *hsp70* loci (Figure 3A and E). We note that the reduced DNA-binding activity of HSF^4 at 33°C was comparable with, based on immunolocalization, or only slightly below, based on EMSA, the wild-type HSF binding at 30°C, which none the less gave clear transcriptional activity. This suggests that the HSF^4 protein is compromised for additional functions related to transactivation.

The mutant HSF was able to respond to recovery from anoxia, an alternative inducer of the heat shock response, at the normal growth temperature of 25°C. When *hsf*^{4} adults or larvae were treated with CO_2 or N_2, HSF became activated and HSP70 synthesis was induced during the recovery period to the same extent as in *hsf*$^+$ animals (Figure 3). Thus, the *hsf*^{4} mutant appears to be temperature-sensitive not only for its function in larval development, but also for its activity in the heat shock response.

Compromised thermotolerance in the hsf^4 *mutant*

Thermotolerance, the ability of the organism to withstand extreme heat stress, correlates well with the induced level of HSP70 expression in *Drosophila* (reviewed in Parsell *et al.*, 1993). For example, a mild heat pre-treatment, which induces HSP synthesis, has been shown to enhance the survival of a subsequent severe heat stress (Mitchell *et al.*, 1979; Velazquez and Lindquist, 1984; Welte *et al.*, 1993). To assess the requirement for HSF in thermotolerance, we compared the ability of *hsf*$^+$ and *hsf*^{4} adults to survive following a severe heat stress. As shown in Figure 4B–D, a clear reduction in survival after a 40 min heat treatment at temperatures of 38°C and above was observed for the *hsf*^{4} mutant when compared either with a wild-type strain (*hsf*$^+$) or a mutant strain carrying two copies of the P[*w*$^+$, *hsf*$^+$] transgene. Thus, HSF is required for thermotolerance in *Drosophila*, presumably through its HSP-inducing activity. Furthermore, in contrast to the *hsf*$^+$ strains, *hsf*^{4} survival did not appear to be affected significantly by a mild heat pre-treatment (20 min at 36°C), suggesting that the beneficial effects of this pre-treatment are also dependent on HSF, and hence are probably conferred by the induced HSPs as proposed previously (Mitchell *et al.*, 1979; Velazquez and Lindquist, 1984; Welte *et al.*, 1993). It is of interest that the loss of HSF function did not affect the ability of adult flies to survive a 40 min heat stress at 37°C (Figure 4A), although an effect on survival was observed for extended periods of stress at this temperature (data not shown). The strong mobilization of HSF activity normally elicited by short exposures to moderate heat stress may, therefore, represent a pre-emptive response in anticipation of more severe stress conditions.

Larval lethality caused by hsf mutations

As mentioned earlier, the lethal stage of all four *hsf* mutations (including *hsf*^{4} at the non-permissive temperature) is 1st or 2nd larval instar. On reaching this stage, mutant larvae die in the ensuing 2–3 days without further growth and development. The mutant larvae did not display any obvious morphologic abnormalities, as judged both by larval cuticle preparations and by examination

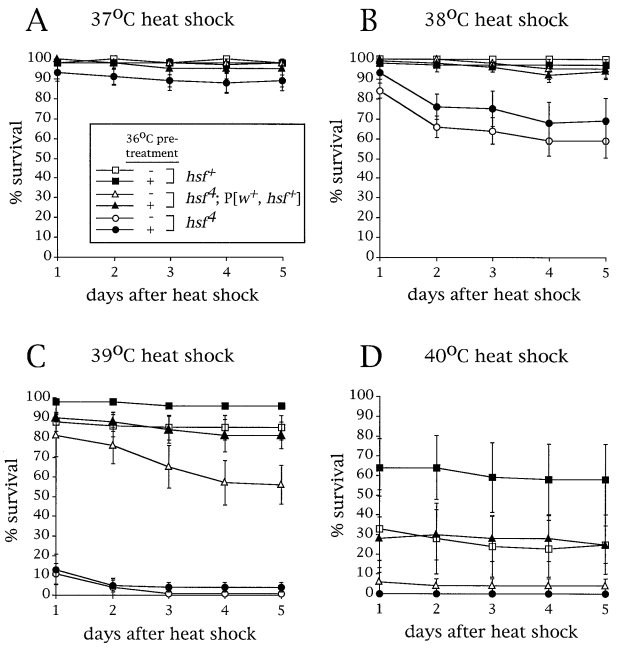

Fig. 4. Thermotolerance of *hsf*^{4} adult flies. Twenty 6- to 10-day-old adults of the indicated genotype were transferred to humidified vials. They were given a heat pre-treatment for 20 min at 36°C as indicated (–/+), allowed to recover for 30 min at 25°C, and subjected to a severe heat stress for 40 min at the indicated temperature (37–40°C). They were then transferred to vials with food and scored for survival over 5 days. Each data point represents the mean of three or four separate experiments performed with different groups of flies; error bars denote the standard error of the mean.

of late embryos stained for tissue markers (horseradish peroxidase for the nervous system and myosin heavy chain for the mesoderm; data not shown). Hence, the larval lethality does not appear to be due to any gross morphogenic defects incurred during embryogenesis.

To examine if other developmental stages have an essential requirement for HSF, we determined the temperature-sensitive lethal period(s) for the *hsf*^{4} mutation. To obtain the latest boundary of any temperature-sensitive period, animals were maintained at 25°C and shifted at progressively later points in development to 29°C, while to obtain the earliest boundary, animals maintained at 29°C were similarly shifted to 25°C. As shown in Figure 5A, the *hsf*^{4} mutant exhibited a single temperature-sensitive period at ~1.5–2.5 days of development, corresponding to the 1st and 2nd larval instars. Upon eclosion, adults were viable and fertile at 29°C. Thus, a requirement for HSF function, as defined by the *hsf*^{4} mutant, appears to be restricted to early larval development.

To be certain that any potential residual activity of the HSF^4 protein at 29°C did not mask a ubiquitous requirement for HSF in every cell, we induced clones homozygous for the null mutation *hsf*^{1} in developing tissues using FLP-FRT-mediated recombination and the *yellow* gene as a marker for the resulting adult structures (Xu and Rubin, 1993). *hsf*^{1} clones induced after the larval lethal stage gave rise to viable populations of cells that were able to form phenotypically normal structures in the adult. Hence, *Drosophila* HSF is not universally required for cell growth or viability under normal conditions. Interestingly, when *hsf*^{1} clones were induced prior to or during the larval lethal stage, they could not be recovered in the head and

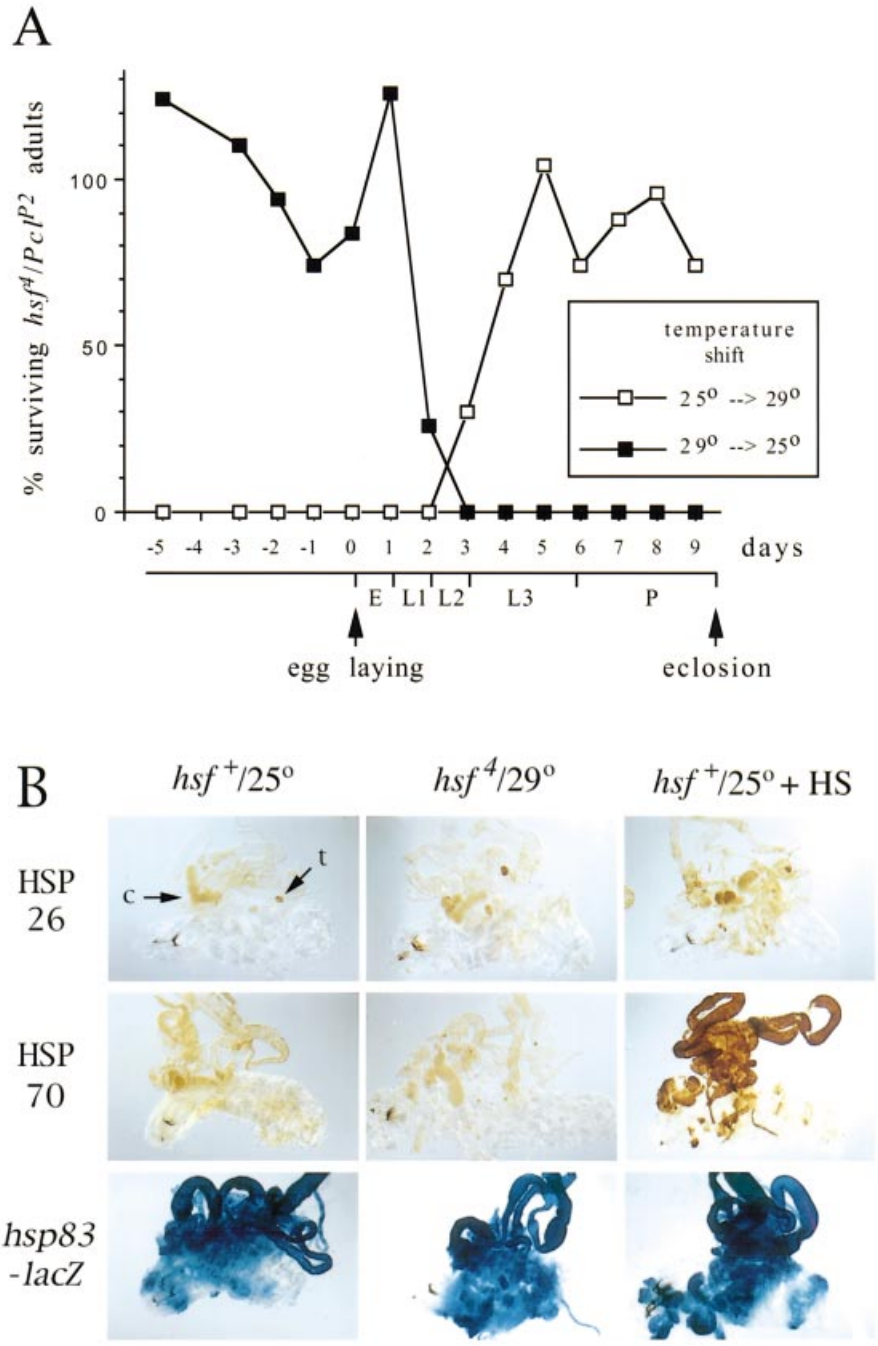

Fig. 5. Temperature-sensitive lethal period and developmental expression of HSPs in the *hsf*^{4} mutant. (**A**) Progeny from the cross of *hsf*^{4}/*hsf*^{4} females to *Pcl*^{P2}/*CyO* males were collected for 24 h periods and temperature-shifted at different times in development as indicated. Developmental age was counted from the end of the collection period; hence, a temperature shift at day 1 refers to animals at 0–1 days of development. Egg laying is set to correspond to day 0 of development. Embryonic (E), 1st to 3rd larval instar (L1–L3) and pupal (P) developmental periods are indicated. The number of *hsf*^{4}/*Pcl*^{P2} adult progeny as a proportion of total adult progeny was scored; when *hsf*^{4}/*Pcl*^{P2} accounted for half of the total progeny, the frequency expected if such animals are viable, survival was considered 100%; hence, values >100% were obtained occasionally due to statistical fluctuation and sample size. The results of one representative experiment are shown. (**B**) *hsf*$^+$ and *hsf*^{4} larvae at 1–3 days of development, at 25 and 29°C respectively, were dissected and stained with antibody (HSP26 and HSP70) or for lacZ activity (*hsp83–lacZ*). In the third column (+HS), *hsf*$^+$ larvae received a 2 h heat shock at 36°C prior to dissection and staining. The central nervous system (c) and testis (t), showing HSP26 and HSP70 immunostaining significantly higher than background, are indicated (HSP70 staining in the testis was observed in other samples not shown). *hsf*$^+$ larvae raised at 29°C did not show noticeably higher levels of HSP expression in the same experiments.

thorax, but gave rise to phenotypically normal structures in the abdomen, suggesting that the HSF requirement in larval development is spatially restricted.

The only known function of HSF is the regulation of HSP synthesis in response to stress. Several HSPs are known to be expressed in differing patterns throughout

Table II. Induction of *hsf* germ-line clones in ovo^{D1} background

Germ-line clones induced	%Fertile females (No./total)
hsf^{+}	100 (14/14)
hsf^{1}	0 (0/52)
hsf^{2}	0 (0/31)
hsf^{3}	0 (0/35)
hsf^{4} (25°C)	100 (52/52)
hsf^{4} (29°C)	100 (45/45)

normal *Drosophila* development in the absence of exogenous stress (reviewed in Arrigo and Tanguay, 1991; Voellmy, 1994). To examine the possibility that the developmental function of HSF might be related to this expression of HSPs, we analyzed the expression of HSP26, HSP70 and HSP83 in hsf^{4} animals during the temperature-sensitive period by staining of dissected larval tissues for HSP protein or *hsp–lacZ* reporter activity. For all of the HSPs tested, the same pattern and level of expression were observed in hsf^{4} mutant larvae at the non-permissive temperature as in hsf^{+} larvae (Figure 5B). As shown previously (Mason *et al.*, 1984; Glaser *et al.*, 1986; Xiao and Lis, 1989), weak expression in the central nervous system and moderate expression in the testis of HSP26 and HSP70 together with strong expression of HSP83 in all tissues were observed relative to heat-induced levels. Similarly, no difference could be detected between hsf^{4} at the non-permissive temperature and hsf^{+} in mRNA levels for the above HSPs by Northern blotting of whole larval RNA preparations (unpublished observations). These results are consistent with published studies indicating that the developmental expression of HSPs is essentially independent of HSEs (Cohen and Meselson, 1985; Hoffman and Corces, 1986; Klemenz *et al.*, 1986; Xiao and Lis, 1989). We conclude, therefore, that the essential developmental function of HSF may involve the regulation of novel, non-heat shock genes.

Arrest of oogenesis for hsf^{1}, hsf^{2} and hsf^{3}, but not hsf^{4}

Immunostaining studies show that HSF is present in the embryo prior to the onset of zygotic gene expression, indicating that maternal HSF is deposited into the oocyte (unpublished observations). To confirm and extend the studies of the developmental role of HSF, we determined the phenotypic effects of completely removing the maternal HSF component by constructing *hsf* mutant germ-line clones using the ovo^{D1}/FLP-FRT technique (Chou and Perrimon, 1992). The dominant female-sterile mutation ovo^{D1}acts in the germ-line to block oogenesis at an early stage; FLP-FRT-mediated recombination is used to remove the ovo^{D1} mutation and simultaneously make homozygous any mutation of interest in a subset of germ-line stem cells (Chou and Perrimon, 1992).

The results of such an experiment for each of the *hsf* mutations are shown in Table II. As might be expected, induction of germ-line clones for hsf^{+} or the hsf^{4} allele at the permissive temperature (25°C) restored fertility to 100% of the resulting mosaic females. Surprisingly, females mosaic for hsf^{1}, hsf^{2} or hsf^{3} germ-line clones were sterile. This sterility was not due to other, cryptic mutations on the *hsf* mutant chromosomes, as fertility was restored by crossing in a single copy of the P[w^{+}, hsf^{+}] transgene. By contrast, mosaic hsf^{4} females were fertile even at the temperature non-permissive for larval development (29°C). hsf^{4} homozygous and hsf^{4}/Pcl^{P2} heterozygous females were also fertile at 29°C. These results indicate a new requirement for HSF in oogenesis not revealed by the conditional hsf^{4} mutant. Furthermore, they imply that the requirements for HSF function in oogenesis and larval development are non-identical. Either the HSF^{4} mutant protein retains a residual amount of activity at 29°C that is sufficient for normal oogenesis but not for larval development, or, alternatively, the hsf^{4} mutation disrupts a function only required in larval development.

When we examined ovaries isolated from mosaic hsf^{1}, hsf^{2} or hsf^{3} females, we were unable to distinguish the *hsf* mutant germ-line clones from the ovo^{D1} background. This indicated that the sterility caused by the *hsf* mutations was due to a block of oogenesis at a stage similar to ovo^{D1} arrest or prior to egg chamber formation. To distinguish between these possibilities, we induced *hsf* germ-line clones by the FLP-FRT technique in a wild-type (ovo^{+}) background. Ovaries from females mosaic for such clones contained a proportion of ovarioles in which egg chambers had apparently arrested development at a pre-vitellogenic stage. When clones were induced using the null allele hsf^{1}, the nurse cells and oocyte of such arrested egg chambers failed to immunostain with HSF antibody, confirming that these egg chambers correspond to hsf^{1} germ-line clones (Figure 6A). Such clones contained a single oocyte and the normal number of 15 nurse cells. The hsf^{1} mutation therefore affects neither germ-line stem cell divisions that yield the 16-cell cyst, nor the differentiation of this cyst into nurse cells and the oocyte. The only observable defect in the hsf^{1} egg chambers was that the DNA content of the nurse cells appeared somewhat low, suggesting a possible defect in the endoreplication that normally occurs in these cells (Spradling, 1993). Thus, HSF is required in the female germ-line for development of the egg chamber, at a stage after formation and differentiation of the germ-line cyst, but prior to vitellogenesis.

The requirement for HSF in oogenesis also does not appear to be related to the regulation of HSP expression. HSP83 is expressed in developing egg chambers (Zimmerman *et al.*, 1983; Ambrosio and Schedl, 1984), but, as shown in Figure 6B, the expression of an *hsp83–lacZ* reporter transgene in hsf^{1} germ-line clones was the same as in corresponding hsf^{+} egg chambers. The timing of expression of the other HSPs synthesized during oogenesis, HSP26 and HSP28 (Zimmerman *et al.*, 1983; Ambrosio and Schedl, 1984), does not coincide with the temporal requirement for HSF; moreover, the expression of HSP26 in the ovary has been shown to be HSE independent (Cohen and Meselson, 1985). Thus, as in larval development, the essential function of HSF in oogenesis appears to be exerted through targets unrelated to *hsp* genes.

Discussion

Extensive biochemical and molecular studies in yeast, fly and mammalian systems have indicated that HSF is a highly conserved stress-regulated transcriptional activator of genes encoding the HSPs. This function of HSF has

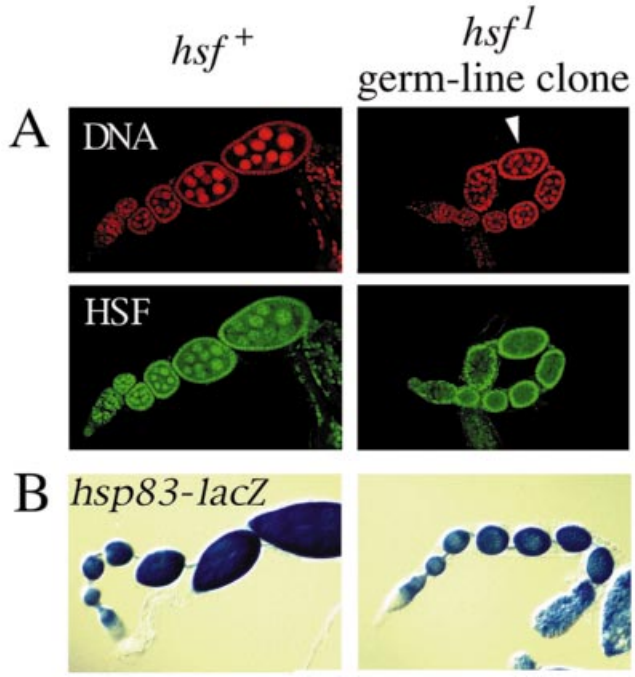

Fig. 6. Characterization of *hsf* mutant germ-line clones. (**A**) Germ-line clones homozygous for the null mutation hsf^1 in a wild-type (ovo^+) background were induced in larvae at 3–6 days of development using FLP-FRT-mediated recombination and analyzed in adult females. DNA was visualized by staining with propidium iodide and HSF by immunostaining with fluorescein-conjugated secondary antibody. Early stages of oogenesis are shown (Spradling, 1993). In the hsf^+ panels, the germarium, containing stem cells and forming egg chambers, is at the lower left and a stage 7–8 egg chamber, commencing vitellogenesis, is at the upper right; each egg chamber is comprised of 15 nurse cells and an oocyte of germ-line origin, and a surrounding layer of follicle cells of somatic origin. hsf^1 germ-line clonal egg chambers were identified by the absence of HSF immunostaining of nurse cell nuclei and the oocyte. In the ovariole shown, both of the germ-line stem cells are clonal for the hsf^1 mutation since none of the nurse cell and oocyte nuclei shows staining. The hsf^1 clones arrest in oogenesis at stage 5–6 (arrowhead) and, after this point, appear to degenerate. In the same experiments, we have failed to detect hsf^1 clones among the somatic follicle cells, suggesting that such clones are inviable and are eliminated from the developing cell population and that HSF is required in these cells as well. This requirement for HSF may be confined to early steps in follicle cell development since clones induced at later points in oogenesis were detected (data not shown). (**B**) Germ-line clones were induced as in (A), but the flies also contained the *hsp83–lacZ* transgene. Ovaries from mosaic females were dissected and stained for lacZ activity. hsf^1 germ-line clonal egg chambers were identified as those displaying the arrest phenotype shown in (A).

been confirmed by genetic studies in yeast, which also revealed an essential requirement for HSF under normal growth conditions. In this report, we have described mutations in the *Drosophila hsf* gene. Like yeast HSF, the mutations demonstrate that *Drosophila* HSF is essential for the heat shock response *in vivo*, but, unlike yeast HSF, the *Drosophila* protein is dispensable for general cell growth or viability under normal conditions. Moreover, the mutations also reveal an unforseen requirement for HSF during oogenesis and larval development.

The temperature-sensitive allele hsf^4 has proven useful in exploring the physiological function of HSF in the heat shock response. Animals carrying this mutation fail to activate HSP expression in response to heat stress at any temperature. The effect of the hsf^4 mutation on HSF function may be pleiotropic. At all heat-shock temperatures, the DNA-binding function of the HSF^4 protein is impaired. The substitution of methionine, which bears a more extended side chain, for Val57 in the hydrophobic core of the DNA-binding domain may compromise the ability of the domain to maintain its overall native structure at elevated temperature by disrupting essential packing interactions. Alternatively, the mutation may perturb only specific functional surfaces of this domain. Interestingly, at the intermediate heat shock temperature of 33°C, the HSF^4 protein binds DNA at a level that might be expected to be sufficient for promoter activation, but still fails to activate transcription. This result suggests that the V57M substitution may also disrupt the transactivation function of HSF by allosterically affecting the availability of the C-terminal activation domain. In yeast, a mutation in the DNA-binding domain that affects transactivation has also been reported (Bonner *et al.*, 1992). The failure of the hsf^4 mutant to induce HSP expression confirms that *Drosophila* HSF is required for HSP synthesis in response to heat stress *in vivo*, consistent with similar findings in *S.cerevisiae* (Smith and Yaffe, 1991).

As a consequence of the lack of HSP induction, the hsf^4 mutation dramatically compromises thermotolerance under conditions of extreme heat stress. This finding suggests strongly that induced HSPs are essential for thermotolerance in *Drosophila*, in agreement with previous studies showing that thermotolerance correlates with HSP70 levels (reviewed in Parsell *et al.*, 1993). Somewhat surprisingly, however, the hsf^4 mutation has no effect on survival from a moderate heat stress (40 min at 37°C). hsf^4 also responds normally under these conditions in standard behavioral assays (bang-sensitivity and countercurrent distribution, testing for neurological function and taxis respectively; Benzer, 1967; Ganetzky and Wu, 1982; unpublished observations), indicating the apparent absence of a general deficit of the nervous system or musculature. Thus, the dramatic induction of HSPs under conditions of moderate stress may not have an immediate function, but may instead represent a pre-emptive response in anticipation of a more severe stress. Moreover, it appears that mechanisms other than enhanced HSP synthesis contribute to thermotolerance under moderate stress conditions. The relatively high level of constitutive HSC70 found in *Drosophila* may provide thermoprotection (Lindquist and Craig, 1988), and additional pathways and mechanisms of thermotolerance reported in other species may turn out to play a role as well (reviewed in Mager and Moradas Ferreira, 1993; Mager and De Kruijff, 1995; see also De Virgilio *et al.*, 1994). A role for yeast HSF in thermotolerance has been suggested previously (Sewell *et al.*, 1995). However, when the constitutive HSP104 level is high, HSF does not appear to be required for thermotolerance in yeast (Smith and Yaffe, 1991; Lindquist and Kim, 1996).

The *hsf* mutants were isolated in genetic screens for recessive lethal mutations under conditions of no external stress, revealing a requirement for HSF function under normal growth conditions in *Drosophila*. Our analyses clearly demonstrate a requirement for HSF in oogenesis and early larval development. The viability of the conditional mutant hsf^4 in other stages and the ability of hsf^1 null mutant clones to develop into phenotypically normal

adult structures indicate that HSF is not universally required for cell growth or viability. Thus, *Drosophila* seems to have dispensed with a requirement for HSF for general cell growth or viability, previously observed in yeast (Sorger and Pelham, 1988; Wiederrecht *et al.*, 1988; Gallo *et al.*, 1993), but has acquired a more specific function for the protein in development.

What might be the function of HSF in *Drosophila* development? The phenotypes of the *hsf* mutations do not reveal defects in specific developmental processes. Both the larval and oogenesis phenotypes appear as a growth or developmental arrest without manifestation of obvious morphogenic defects. As early larval development and early stages of development of the egg chamber, following its formation in the germarium, represent processes marked by cell growth and chromosomal endoreplication, and a paucity of cell proliferation and differentiation (Ashburner, 1989; Spradling, 1993; Demerec, 1994), HSF might be involved in regulating genes related to these processes. Since the HSF requirements for larval development and oogenesis are not identical, the targets of regulation may not be the same. The identity of the target genes is not known. Our results argue against *hsp* genes as targets since the normal developmental expression of HSPs during the periods of HSF requirement is unaffected by loss of HSF function. Consistent with this finding, we have not detected genetic interactions between *hsf* mutations and genetic deficiencies that delete *hsp* loci (unpublished observations). Thus, the essential physiological function of HSF under normal conditions appears to be the regulation of novel, non-*hsp* genes involved in presently unknown growth or developmental processes.

The developmental phenotypes of hsf^3, a mutation in the recognition helix of the DNA-binding domain, imply that the function of HSF in larval development and oogenesis requires its DNA-binding activity. However, since HSP expression remains unchanged when HSF function is required, developmentally active HSF is unlikely to be activated in the same way as when induced by heat stress. Given that the affinity of the HSF monomer for DNA is low (Westwood *et al.*, 1991; Kim *et al.*, 1994), HSF could become partially activated to a trimeric form with high affinity for the HSE, but devoid of the capacity for transcriptional activation. Such an intermediate form of HSF previously has been proposed to exist in cells treated with salicylate (Jurivich *et al.*, 1992). Alternatively, monomeric HSF normally present in the cell might be recruited to specific, non-*hsp* promoters by another DNA-binding protein as part of a heteromeric complex. HSF could then activate or repress gene expression, depending on the promoter context. Again, owing to the non-identical requirements for HSF in larval development and oogenesis, HSF could act by different mechanisms for these two processes.

The availability of mutant alleles of *Drosophila hsf*, especially the conditional hsf^4 mutation, should allow the identification of other components of the developmental HSF pathway through screens for genetic interactions and analyses of differential gene expression. The elucidation of this pathway may also provide clues to the potentially important role of HSF2 in vertebrate development. In addition, these mutations should facilitate further study of the function and regulation of HSF in the heat shock response and the role of this response in the adaptation of organisms to environmental and pathophysiologic stresses.

Materials and methods

Single-embryo PCR and hybridization in situ to polytene chromosomes

Embryos from the cross $Pcl^{P2}/+ \times Pcl^{P2}/+$ were collected overnight on egg collection plates, dechorionated for 3 min in 50% Chlorox bleach, placed individually into 0.5 ml PCR tubes and frozen at –80°C. They were then macerated with a sterile, aerosol-proof pipet tip, 12.5 µl of lysis buffer [1× Taq buffer (Promega), 1.5 mM $MgCl_2$, 1% Tween, 1% NP-40, 0.2 mg/ml proteinase K] were added and the suspension was incubated overnight at 65°C. The resulting lysate was incubated at 95°C for 15 min to inactivate the proteinase K and was frozen at –20°C. For analysis, 1–2 µl of embryo lysate were added to a 25 µl standard PCR using *Taq* polymerase (Promega) and amplified for 40 cycles with primers to exon 4 of the *hsf* gene (Clos *et al.*, 1990) and exon 3 of the *RPII140* gene (Falkenburg *et al.*, 1987) in the same reaction.

Polytene chromosome squashes from $Pcl^{P2}/+$ larvae were done as described (Ashburner, 1991). Biotinylated *hsf* probe was prepared by random prime labeling an *Eco*RI *hsf* cDNA fragment (Clos *et al.*, 1990) using the BioPrime DNA Labeling System (Gibco-BRL). Hybridization *in situ* and detection were as described (Gong *et al.*, 1995). Samples were analyzed on a Zeiss Axiophot microscope.

Ethyl methanesulfonate (EMS) mutagenesis and screening

Three-day-old adult male flies were fed EMS (Lewis and Bacher, 1968) and were then used in two F_2 lethal screens. In the first screen, 1851 progeny from flies carrying an unmarked isogenic 2nd chromosome were screened for lethality in *trans* to Pcl^{P2} at 25°C. In the second screen, 3138 progeny from flies carrying an isogenic *dp cl cn bw* chromosome were screened in *trans* to Pcl^{P2} at 29°C. All putative mutations were tested further against the larger deficiency *Df(2R)Pcl11B* and three alleles of *Pcl* (Pcl^{I3}, Pcl^{I5}, Pcl^{E90}; Lindsley and Zimm, 1992). Mutations lethal in *trans* to *Df(2R)Pcl11B*, but able to complement *Pcl*, were analyzed further as candidate *hsf* mutations.

Genomic hsf sequencing and rescue

The *hsf* gene (Clos *et al.*, 1990 and unpublished) was amplified in overlapping 1–2 kb fragments using *Pfu* polymerase (Stratagene), under standard reaction conditions, from DNA (10–50 ng/100 µl reaction) isolated from adult flies heterozygous for the candidate *hsf* mutations over their respective isogenic 2nd chromosomes, or, in the case of the hsf^4 mutation, from hsf^4/Pcl^{P2} hemizygotes. A reaction without DNA template was always included to be sure that potential contaminating DNA was not being amplified. The products were analyzed on an agarose/1× Tris/acetate/EDTA gel, purified using the Geneclean II kit (Bio101), and sequenced with internal primers using the Sequenase 2.0 kit (USB) with slight modification of the manufacturer's protocol. The sequence obtained from candidate *hsf* mutant flies was compared with the unmutagenized isogenic strain. Mutations were confirmed by sequencing PCR-amplified DNA from dead homozygous hsf^1, hsf^2 and hsf^3 larvae, and DNA from hsf^4/Pcl^{P2} hemizygous adults, amplified with different primers.

A rescue plasmid was constructed by subcloning an 8 kb *Hin*dIII fragment containing the *hsf* gene from the genomic clone EMBL3-104 (Clos *et al.*, 1990) into the pCaSpeR4 P-element transformation vector (Pirrotta *et al.*, 1985), to yield P[w^+, hsf^+]. This construct was injected into embryos along with a transposase source as described (Rubin and Spradling, 1982).

Electrophoretic mobility shift assay (EMSA), Western blot analysis and dot-blot analysis

For stress response experiments, adult flies were collected the day before the experiment (40 flies/vial) and prior to treatment were transferred without anesthesia to vials without food containing a moistened piece of filter paper; larvae were collected just prior to treatment (10 larvae/tube) in 5 ml Falcon 2058 tubes. Stress treatments were administered as described below. Following treatment, adult flies were shaken into 50 ml Falcon 2070 tubes pre-cooled on dry ice; tubes with larvae were placed directly in dry ice. The frozen adults or larvae were then transferred to 1.5 ml Eppendorf tubes.

To prepare protein extracts for analysis, the animals were transferred to a 4 ml AA Thomas smooth pestle tissue grinder, 0.4 ml (adults) or

0.2 ml (larvae) of extraction buffer [10 mM HEPES (pH 7.9), 0.4 M KCl, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT) and 5% glycerol supplemented with 1 mg/ml each of aprotinin, leupeptin and pepstatin A and 100 μg/ml each of PMSF and AEBSF] was added, the samples were homogenized for 10 strokes and were transferred to 1.5 ml Eppendorf tubes. They were then subjected to three cycles of freeze (dry ice for 5 min)–thaw (ice for 30 min), and were centrifuged for 5 min at 14 000 r.p.m. at 4°C. The supernatants were transferred to 1.5 ml Beckman polyallomer tubes and centrifuged for 10 min at 40 000 r.p.m. in a Beckman TLA-45 rotor at 4°C. The resulting supernatants were frozen at –80°C.

EMSA was carried out by incubating 20–25 μg of extract protein in extraction buffer (total volume adjusted to 5 μl) with 5 μl of binding buffer [15 mM Tris, pH 7.4, 0.1 mM EGTA, pH 8.0, 0.5 mM DTT, 5% glycerol, 1 mg/ml poly(dI·dC)–poly(dI·dC) (Pharmacia), 25–100 fmol of ^{32}P-labeled HSE oligonucleotide probe (three nGAAn repeats, Wisniewski *et al.*, 1996)] for 15 min on ice. The reactions were analyzed on a 0.8% agarose/0.5× Tris/borate/EDTA gel.

Western blot analysis of extracts was performed as described (Rabindran *et al.*, 1994), using antibody to *Drosophila* HSF (Westwood *et al.*, 1991) and to *Drosophila* HSP70 (Velazquez *et al.*, 1983). Typically, 10–15 mg of extract protein were analyzed per sample. Detection was by enhanced chemiluminescence (ECL; Amersham).

RNA was isolated from animals using the TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. Three μg of total RNA were dot-blotted to a nylon membrane (Genescreen, NEN) and hybridized with radiolabeled *hsp70* probe as described (Krawczyk and Wu, 1987; Tsukiyama *et al.*, 1994).

Stress treatments and thermotolerance experiments

All heat treatments were carried out in a Techne Hybridizer HB-1D oven; the precise temperature inside the vials was monitored using a YSI model 46TUC Tele-thermometer. For anoxia experiments, CO_2 or N_2 was administered through tubing fed into the vials.

For thermotolerance experiments, twenty 0- to 5-day-old adult flies were collected per vial, aged 5–6 days, and prior to treatment were transferred to vials without food containing a moistened piece of filter paper. The relative positions of vials in the Hybridizer oven were varied in different experiments in order to eliminate the effect of small (0.2°C or less) temperature gradients inside the chamber.

HSF immunostaining

Groups of 10 late third instar larvae were placed in 5 ml Falcon 2058 tubes sealed with cotton and were subjected to stress, as described above. Salivary glands were then dissected out in phosphate-buffered saline (PBS) and placed in fixative (50% acetic acid/3.7% formaldehyde) for 5 min. Preparation of chromosome spreads and HSF immunostaining were essentially as described (Westwood *et al.*, 1991); DNA was stained with 1 mg of propidium iodide/ml of PBS. Samples were analyzed on a Nikon Optiphot microscope.

Ovaries were dissected from cold-anesthetized adult females in PBS, ovarioles were separated using minn pins (Carolina Science Materials), and the tissues were fixed in 15 mM KH_2PO_4/K_2HPO_4 (pH 6.8), 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$, 6% formaldehyde (Montell *et al.*, 1992), containing 0.01% Tween-20. After two 30 min washes in PBS/0.1% Tween (PBST), the tissues were incubated in 0.4 mg of RNase/ml of PBS at room temperature for 2 h (Orsulic and Peifer, 1994), washed twice for 5 min in PBST, treated with methanol for 20 min, and washed again. The tissues were blocked with PBST/10% normal donkey serum (NDS; Jackson Immunoresearch Laboratories) for 1–3 h and then incubated overnight at 4°C with HSF antibody (Westwood *et al.*, 1991) diluted 1:500 in PBST/5% NDS. After washing twice for 10 min with PBST, biotinylated goat anti-rabbit antibody (Vector Laboratories), diluted 1:200 in PBST, was applied for 2–3 h. The tissues were again washed twice for 10 min in PBST and were incubated with avidin–FITC (Vector Laboratories), diluted 1:200 in PBST, for 2–3 h. The tissues were then stained with 10 mg of propidium iodide/ml of PBST for 20–30 min, washed four times for 30 min in PBST, and mounted on slides in 'anti-fade' (1 mg/ml phenylenediamine in 70% glycerol/PBS); prior to application of cover slips, ovaries were dissected further, as above, to better separate ovarioles for microscopic analysis. The samples were analyzed on a Bio-Rad MRC-1024 laser scanning confocal imaging system.

Analysis of temperature-sensitive period and developmental HSP expression

Developmental experiments with the *hsf*^{4} mutant were carried out in a forced-air incubator where the temperature fluctuated by ~0.5°C. During the initial genetic screening, the temperature range was ~28.2–28.7°C. This temperature was non-permissive to development of *hsf*^{4}/*Pcl*^{P2} heterozygotes, but allowed a small number of *hsf*^{4} homozygotes to survive to the adult stage. To determine the temperature-sensitive lethal period(s) of the *hsf*^{4} mutation, *hsf*^{4}/*Pcl*^{P2} heterozygotes were analyzed at this temperature, which gave better overall culture viability. For HSP expression experiments (below), a slightly higher temperature (~28.5–29.0°C) that was non-permissive to development of *hsf*^{4} homozygotes was used.

For analysis of HSP protein expression, embryos were collected on egg collection plates with yeast paste for 2 days at 25 or 29°C and the collections were aged for 1 day at the same temperature. Approximately 20 of the resulting larvae, representing organisms at 1–3 days of development, were dissected in PBST using tungsten needles, and were fixed and immunostained using *Drosophila* HSP26 (1:100 dilution; Marin *et al.*, 1993) or *Drosophila* HSP70 (1:500 dilution; Velazquez *et al.*, 1983) antibody as described for eye imaginal disks (Van Vactor *et al.*, 1991), except that the glutaraldehyde post-fixation step was omitted. For analysis of *hsp83–lacZ* expression, a line transgenic on the X chromosome for the full-length *hsp83* promoter driving *lacZ* expression was used (line c83Z.-880; Xiao and Lis, 1989). For determination of *lacZ* expression, larvae or ovaries were fixed for 15 min in 0.5% glutaraldehyde in PBS, and were washed and stained with X-Gal as described (Hursh *et al.*, 1993). Samples were analyzed on a Zeiss Axiophot microscope.

Somatic and germ-line clone experiments

hsf mutant somatic clones in the adult cuticle and germ-line clones in the ovary were generated using the FLP-FRT techniques adapted for the 2nd chromosome (Xu and Rubin, 1993 and Chou and Perrimon, 1992, respectively). To induce FLP activity, animals were heat-shocked for 2 h at 37°C in a Techne Hybridizer HB-1D oven.

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