Genetic Analysis of Viable Hsp90 Alleles Reveals a Critical Role in Drosophila Spermatogenesis

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ABSTRACT

The Hsp90 chaperone protein maintains the activities of a remarkable variety of signal transducers, but its most critical functions in the context of the whole organism are unknown. Point mutations of *Hsp83* (the Drosophila Hsp90 gene) obtained in two different screens are lethal as homozygotes. We report that eight transheterozygous mutant combinations produce viable adults. All exhibit the same developmental defects: sterile males and sterile or weakly fertile females. We also report that *scratch*, a previously identified male-sterile mutation, is an allele of *Hsp82* with a *P*-element insertion in the intron that reduces expression. Thus, it is a simple reduction in Hsp90 function, rather than possible altered functions in the point mutants, that leads to male sterility. As shown by light and electron microscopy, all stages of spermatogenesis involving microtubule function are affected, from early mitotic divisions to later stages of sperm maturation, individualization, and motility. Aberrant microtubules are prominent in yeast cells carrying mutations in *HSP82* (the yeast Hsp90 gene), confirming that Hsp90 function is connected to microtubule dynamics and that this connection is highly conserved. A small fraction of Hsp90 copurifies with taxol-stabilized microtubule proteins in Drosophila embryo extracts, but Hsp90 does not remain associated with microtubules through repeated temperature-induced assembly and disassembly reactions. If the spermatogenesis phenotypes are due to defects in microtubule dynamics, we suggest these are indirect, reflecting a role for Hsp90 in maintaining critical signal transduction pathways and microtubule effectors, rather than a direct role in the assembly and disassembly of microtubules themselves.

HEAT-SHOCK proteins (Hsp's) play roles in an extraordinary variety of normal cellular processes, including protein trafficking, signal transduction, DNA replication, and protein synthesis (Hartl 1996). However, our understanding of the most critical *in vivo* functions of Hsp's is still limited, and the roles played by heat-shock proteins in the development of multicellular organisms are just beginning to be unveiled (Cutforth and Rubin 1994; Parsell and Lindquist 1994; van der Straten *et al.* 1997).

Hsp's comprise several highly conserved, distinct families of proteins. The Hsp 90 family includes proteins encoded by the *Hsp90* and *GRP94* genes of human, mouse, and chicken cells, the yeast *HSP82* and *HSC82* genes, the *HtpG* gene of bacteria, and the *Hsp83* gene of Drosophila. (Following conventional usage, we refer to the Drosophila protein as Hsp90, and the gene as *Hsp83*.) In addition to being induced by heat, most Hsp90 proteins are constitutively expressed and induced in response to certain developmental signals.

Deletion of the *Escherichia coli* Hsp90 homolog HptG produces only subtle growth defects (Bardwell and Craig 1988). But in the budding yeast *Saccharomyces cerevisiae*, cells that lack Hsp90 die at all temperatures tested and the quantity of Hsp90 required for growth increases as the temperature increases (Borkovich *et al.* 1989). Hsp90 is also essential in the only other eukaryotes in which it has been tested, *Drosophila melanogaster* (Cutforth and Rubin 1994; van der Straten *et al.* 1997) and *Schizosaccharomyces pombe* (Aligue *et al.* 1994). Although the bacterial Hsp90 (HtpG) protein cannot rescue yeast cells that lack Hsp90, the human Hsp90 (Picard *et al.* 1990; Minami *et al.* 1994) and Drosophila Hsp90 (L. Yue and S. Lindquist, unpublished results) proteins not only rescue Hsp90-deficient *S. cerevisiae* cells from lethality, but also support rapid growth. Thus, all functions of Hsp90 that are essential in *S. cerevisiae* are conserved in these distant eukaryotic lineages.

Members of the Hsp90 family associate with many other cellular proteins, including steroid hormone receptors (Bohen and Yamamoto 1994), oncogenic tyrosine kinases (Brugge 1986), the serine/threonine ki-

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nase c-Raf (Stancato *et al.* 1993), the heme-regulated eIF-2-alpha kinase (Rose *et al.* 1989), casein kinase II (CKII; Miyata and Yahara 1992, 1995), calmodulin (Minami *et al.* 1993), actin (Nishida *et al.* 1986), and tubulin (Redmond *et al.* 1989). *In vitro*, at very high protein concentrations, Hsp90 functions as a general molecular chaperone, preventing the aggregation of unstable or denatured proteins (Jakob *et al.* 1995; Bose *et al.* 1996; Freeman *et al.* 1996; Kimura *et al.* 1997). *In vivo*, however, Hsp90 chaperone functions are required only with proteins that are destabilized by heat or have difficulty reaching stable conformations at normal temperatures (Nathan *et al.* 1997). Most notably, the latter include a variety of proteins involved in signal transduction. Data from several laboratories indicate that Hsp90 maintains these signal transducers in inactive, but readily activatable states in fungi (Picard *et al.* 1990; Xu and Lindquist 1993; Nathan and Lindquist 1995) and animals (Pongratz *et al.* 1992; Nordeen *et al.* 1994; Antonsson *et al.* 1995; Enan and Matsumura 1995; Holley and Yamamoto 1995; Kimura *et al.* 1995; Jaiswal *et al.* 1996; Jaskoll *et al.* 1996; Louvion *et al.* 1996; Pettersson *et al.* 1996). A curious aspect of Hsp90 function in such pathways is the paradox between the breadth and the specificity of its functions: it functions with transducers as diverse as glucocorticoid and dioxin receptors, PIP2-dependent phospholipase D, and Raf kinase, yet the Hsp90 dependence of even closely related proteins varies widely. For example, v-src is far more dependent on Hsp90 than c-src for activity (Xu and Lindquist 1993; Kimura *et al.* 1995; Dey *et al.* 1996; Xu *et al.* 1999). Hsp90 does not recognize its targets through specific elements, but rather through structural features of their folding intermediates. The inherent conformational instability of signal transducers likely facilitates the conformational changes they employ to regulate growth and development. Presumably, small differences in the stability of the folded states of these proteins and the structures of their folding intermediates dictate the degree to which they are dependent upon Hsp90.

Consistent with a role for Hsp90 protein in maintaining the structure and function of key elements in signal transduction pathways, Drosophila *hsp83* mutations were recovered in screens designed to identify enhancers of *Sevenless* and suppressors of *Raf* (Cutforth and Rubin 1994; Dickson *et al.* 1996; van der Straten *et al.* 1997). Moreover, when the *hsp83* mutations isolated in these screens were crossed into diverse genetic backgrounds, a small percentage (~1–2%) of adult progeny exhibited developmental abnormalities (Rutherford and Lindquist 1998). The abnormalities appearing in *Hsp83* heterozygotes were diverse and, in different flies, virtually every structure could be affected. Each particular abnormality was specific to a different genetic background and further analysis revealed that they arose from hidden, preexisting genetic variation in the stocks, which sensitized different developmental pathways in different flies to the reduction in Hsp90.

These studies reveal a capacity for Hsp90 to function in a wide variety of morphogenetic processes. However, because the defects were associated with mutant or variant morphogenetic elements in the stocks, they do not tell us whether Hsp90 normally functions in these pathways, nor do they reveal the most critical general pathways that depend upon this chaperone.

The lethality of all known *hsp83* mutations makes determining its most critical functions difficult. We have discovered that several of the *hsp83* mutants, isolated in the above-mentioned screens, are viable as transheterozygotes. Moreover, a previously characterized viable mutation that mapped to the vicinity of *hsp83* (Castrillon *et al.* 1993) is a *P*-element insertion that reduces the level of Hsp90 expression. Our analysis demonstrates a critical function for Hsp90 during germline development in Drosophila. Spermatogenesis, in particular, is the developmental process most sensitive to the loss of Hsp90 function. Light and electron microscopic analyses of testes suggest that Hsp90 plays a critical role in microtubule dynamics. Moreover, defects in tubulin distributions in yeast *hsp82* mutants indicate this function is highly conserved. Analysis of the association of Drosophila Hsp90 with microtubules suggests that if Hsp90 affects microtubule dynamics it does so indirectly by altering the activities of the regulators of microtubule function.

MATERIALS AND METHODS

Fly stocks and genetic crosses: All wild-type flies used in this study were *D. melanogaster* Oregon R. The *Hsp83* gene has been mapped to cytological position 63 B11-C1. The *hsp83* mutant stocks were obtained from two sources. Enhancers of *Sevenless* were provided by T. Cutforth and G. Rubin (Cutforth and Rubin 1994). Suppressors of activated *Raf* (*Raf*^{torY9}) were obtained from E. Hafen (van der Straten *et al.* 1997). Consistent with common practice, we refer to the alleles of *hsp83* according to their known amino acid substitutions; thus, R48C, a substitution of cysteine for arginine at amino acid 48, is referred to as R48C, 3A as S574C, 4A as S655F, 6A as S592F, 1D as S38L, 6D as E317K, and 9J1 as E377K.

A *P*-element insertion allele of *hsp83* (P582) was recovered initially by lack of complementation with the *hsp83* mutations that act as suppressors of activated *Raf* (van der Straten *et al.* 1997; A. van der Straten, B. Dickson and E. Hafen, personal communication). The *P*-element insertion site in this mutant allele (P582) was found to reside in the first exon of *hsp83* gene (A. van der Straten, B. Dickson and E. Hafen, personal communication). The lethality reverted when the inserted *P* element was excised (L. Yue and S. Lindquist, unpublished results). The stock corresponding to the male-sterile allele of *hsp83, scratch*, was obtained from the stock center at Indiana University. Visible mutations and balancer chromosomes are as described (Lindsley and Zimm 1992).

All genetic crosses were performed at room temperature. Viability of homozygous (mutant 1/mutant 1) or transheterozygous (mutant 1/mutant 2) *hsp83* flies was compared with the viability of heterozygous (mutant/wild-type) flies with similar

genotypes. To test for sterility, three to five males (or females) were crossed to an equal or greater number of virgin wild-type females (or wild-type males), with at least six tests performed on each genotype. Crosses were designated as male (or female) sterile if they produced no progeny, and as weakly fertile if they produced fewer than 20% of the number of progeny produced by mating heterozygous siblings.

Yeast strains: All yeast strains used were derivatives of W303 (*ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1*: R. Rothstein). The diploid strains iP82 and iG170D were produced from ΔPCLD, a diploid with both copies of *HSP82* and of *HSC82* disrupted, *i.e.*, rescued from the lethality of these disruptions by a wild-type *HSC82* expression plasmid (Nathan and Lindquist 1995). Plasmids piHGpd/P82 and piHGpd/G170D (Nathan *et al.* 1997), containing, respectively, wild-type or *G170D* mutant sequences under the control of the strong constitutive GPD promoter, were linearized with *Nhe*I and transformed into ΔPCLD. Transformants were grown on 5-fluoroorotic acid media [1.7 g yeast nitrogen base without amino acids or ammonium sulfate per liter; 1 g proline/liter; 20 g glucose/liter supplemented with essential amino acid and nutrients (McCusker and Davis 1991)] to select for loss of the wild-type *HSC82* expression plasmid.

Polymerase chain reaction (PCR) experiments: Genomic DNA from wild-type or *scratch* adult flies was isolated as described (Ashburner 1989). A primer, specific to the *P*-element termini (Pend: CGA CGG GAC CAC CTT ATG TTA TTT CAT C) was used with two Hsp90-specific primers (P1: TGG GCT TTG CTC AAT CGT TCG GAC CAC T; P2: GGA TCA CCT TTA GGA CCT TGT TCT GCT G) for PCR reactions.

Protein analysis in Drosophila tissues: Developmentally staged embryos, larvae, and pupae were collected and washed with distilled water. Testes and ovaries from adult males and females were hand dissected in Ringer's buffer (130 mm NaCl, 5 mm KCl, 2 mm $CaCl_2$, and 10 mm Hepes at pH 6.9). Embryos, larvae, pupae, testes, ovaries, and the remaining body of adult flies (minus testes or ovaries), were placed in Eppendorf tubes in a solution of 1× phosphate-buffered saline (PBS) with 0.1% Triton X-100. Samples were frozen in liquid nitrogen, ground with a pestle, and refrozen in liquid nitrogen after ~1 min. This freeze/thaw process was repeated five times. Samples were spun in a microfuge for 30 sec, supernatants were recovered, and protein concentrations were determined by the BCA method (Bio-Rad, Richmond, CA). Protein samples were mixed with 2× sample buffer (4% SDS, 20% glycerol, 10% 2-mercapto-ethanol), and heated at 95° for 2 min. Proteins (10 μg) were electrophoretically separated on 7.5% SDS gels and transferred to Immobilon (Millipore, Bedford, MA).

A monoclonal antibody specific for Drosophila Hsp90 (3F6, gift of Dr. R. Tanguay; Carbajal *et al.* 1990) was used at a dilution of 1:40,000; a monoclonal antibody specific for tubulin (Dm1a; Amersham, Arlington Heights, IL) was used at 1:2000; a monoclonal antibody that recognizes both Hsp70 and its constitutive relatives (7.10; Velazquez *et al.* 1983) was used at 1:1000; a polyclonal antibody specific for TATA-binding protein (TBP; gift of Dr. J. Kadonaga, University of California, San Diego) was used at 1:2000. Immunodetection was performed using either ECL (Amersham) according to the manufacturer's instructions, or radioactive-labeled [^{125}I]-protein A (Amersham) with phosphor-image analysis (Molecular Dynamics, Sunnyvale, CA) for quantification.

Purification of microtubule proteins: Microtubules were purified from Drosophila embryos by taxol-induced assembly (Goldstein *et al.* 1986) or by repeated cycles of assembly and disassembly as described (Olmsted and Borisy 1973; Srinivasan and Karr 1995). Fractions of each stage of the purification were analyzed on SDS-PAGE, immunoblotted, and probed using specific antisera as described above.

Morphological examination of testes by microscopy: Testes from males <1 wk old were dissected in Ringer's buffer. For transmission electron microscopy, testes were fixed and processed as described (Yue and Spradling 1992). For light microscopy, testes were torn open and gently squashed under the weight of a cover slip as described (Fuller 1993). Great care was taken to distinguish common squashing artifacts from mutant phenotypes. Only defects that were consistently observed in heteroallelic *hsp83* mutants but not in wild-type controls prepared at the same time and in the same manner are reported. Spermatogenic cells at various stages were visualized by phase-contrast microscopy. For immunostaining, squashed testes on slides were frozen in liquid nitrogen, thawed, fixed with 4% formaldehyde in 1× PBS, dehydrated by ethanol, and immunologically stained as described (Graner *et al.* 1994).

The monoclonal antibody 3F6 was used at a 1:2000 dilution; a polyclonal rabbit serum against Drosophila Hsp90 (gift of Dr. J. Bonner, Indiana University) was used at a 1:1000 dilution; monospecific anti-DROP-1 antibody was used at a 1:10 dilution (Graner *et al.* 1994). Immune complexes were visualized with Texas red-, or FITC-conjugated AffiniPure goat anti-mouse IgG (or anti-rabbit IgG, or anti-rat IgG) from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The stained testes were incubated in 1× PBS containing 1 μg/ml Hoechst dye 22578 for at least 5 min for DNA staining and then mounted in PBS buffer with 50% glycerol and 1 μg/ml antiquenching agent, phenylenediamine. Slides were viewed on a Zeiss (Thornwood, NY) Laser Scan Microscope, a confocal microscope equipped with a UV laser to visualize DNA stained by Hoechst dye. Images were collected using Zeiss LSM software and processed using Adobe Photoshop.

Immunofluorescent staining of yeast cells: Immunofluorescence of yeast cells was performed by a variation of the method of Pringle *et al.* (1991). Cells were grown to 5×10^6 cells/ml in YPD media at 25° and then transferred to YPD media prewarmed to either 25 or 37° for 3 hr. Formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) was added to a final concentration of 4% and the cells were incubated for 1 hr at room temperature. The fixed cells were recovered by centrifugation (2000 rpm for 5 min), washed once with buffer B (0.1 m potassium phosphate, pH 6.8, 0.5 mm $MgCl_2$), washed once with buffer C (Buffer B + 1.2 m sorbitol), and resuspended in 1 ml buffer C. The cell wall was permeabilized by incubation with Zymolyase T-100 (50 μg/ml) and 2-mercapto-ethanol (0.5%) for 30 min at room temperature. Cells were recovered by centrifugation (500 rpm for 7 min), resuspended in buffer C, and attached to polylysine-coated slides. The cells were further permeabilized by incubation in PBS containing 0.2% Triton X-100 for 10 min at room temperature, washed three times with PBS, and incubated in blocking solution (PBS containing 5% sheep serum, 5% fetal calf serum, and 0.5% BSA) for 10 min at room temperature. The cells were then incubated overnight at room temperature with tubulin-specific antisera (TAT1, gift of Dr. S. Uzawa and Dr. H. Joshi of Emory University, diluted 1:50 in blocking solution) and washed four times with PBS and twice for 5 min with blocking solution. They were then incubated for 1 hr at room temperature with Texas red-conjugated F(ab′)2 affinity-purified sheep anti-mouse IgG (Cappel; diluted 1:500 in blocking solution) and washed as before. Finally, the cells were incubated with 4,6-diamidino-2-phenylindole (1 μg/ml) for 15 min at room temperature and washed four times with PBS.

RESULTS

***hsp83* mutations impair germline development:** Each of the seven point mutations of *hsp83* used in this study

TABLE 1

Viable transheterozygotes of *hsp83* mutants

Mutant allele	S38L	R48C	E317K	E377K	S574C	S592F	S655F
S38L	—	ms, fwf	—	ms, fwf	—	—	—
R48C	ms, fwf	—	—	—	ms, fwf	ms, fs	—
E317K	—	—	—	ms, fwf	—	—	—
E377K	ms, fwf	—	ms, fwf	—	ms, fwf	ms, fwf	ms, fwf
S574C	—	ms, fwf	—	ms, fwf	—	—	—
S592F	—	ms, fs	—	ms, fwf	—	—	—
S655F	—	—	—	ms, fwf	—	—	—

The transheterozygous progeny from a cross between two *hsp83* balanced stocks were identified by the lack of dominant visible markers carried on the balancer chromosomes and were tested for fertility. Horizontal row, allele of female heterozygotes; vertical row, allele of male heterozygotes. The phenotypes of the transheterozygous progeny depended only on the genotype of the zygote. ms, male sterile; ff, female fertile; fwf, female weakly fertile; fs, female sterile; a dash indicates lethal.

is homozygous lethal (Cutforth and Rubin 1994; Dickson *et al.* 1996; van der Straten *et al.* 1997). However, when we crossed these mutations *inter se* (Table 1), 8 out of 21 transheterozygous combinations developed to adulthood. (Alleles are here designated according to their known amino-acid substitutions; *e.g.*, R48C is a substitution of cysteine for arginine at amino acid 48.) Of the 8 viable transheterozygotes, 1 exhibited severely reduced viability and another had somewhat reduced viability (<1% and 18% mutant adults emerged *vs.* the expected 33%, Table 2). The other 6 crosses were fully viable, producing transheterozygous adult progeny at expected genotypic frequencies. Remarkably, although the mutations involved are scattered throughout the *hsp83* gene, all 8 viable transheterozygotes exhibited the same phenotype, producing sterile males and either sterile or weakly fertile females (Table 1).

***scratch* is an allele of *Hsp83*:** Previously identified male-sterile mutants that have been mapped to the vicinity of *hsp83* (cytological location: 63 B11-C1) were tested for their ability to complement the *hsp83* mutations. All except *scratch* (Castrillon *et al.* 1993) complemented the *hsp83* mutations (Table 3), indicating they are located in separate genes. *scratch* did not complement three of the seven point mutations of *hsp83* for viability, and failed to complement the remainder for fertility (Table 3). An independently isolated homozygous lethal *hsp83* mutation, *P582*, that has a *P* element inserted into the exon 1 (A. van der Straten, B. Dickson and E. Hafen, personal communication), fails to complement any of the *hsp83* point mutations for viability (data not shown). However, *P582* did complement *scratch* for fly viability and female fertility but not male sterility (Table 3). Thus, *scratch*, P582, and the *hsp83* point mutations define a locus that comprises a single complementation group.

scratch was obtained in a single *P*-element insertional mutagenesis screen (Karpen and Spradling 1992; Castrillon *et al.* 1993). We employed PCR to determine if the *P* element is located in *hsp83*. The position of the *P* element was determined by examining the sizes of amplified products from reactions using primers within

TABLE 2

Viability of *hsp83* transheterozygous mutants

Cross	$+/mut^1$ and $+/mut^2$	mut^1/mut^2	%
S38L × R48C	474	223	32
S38L × E377K	181	90	33
R48C × S574C	375	82	18
R48C × S592F	>1000	6	<1
E317K × E377K	361	140	28
E377K × S574C	329	134	29
E377K × S592F	246	137	36
E377K × S655F	307	125	29

The progeny from a cross between two balanced *hsp83* stocks are expected in a 1:1:1:1 ratio. Flies that receive two balancer chromosomes die because balancer chromosomes carry recessive lethal markers. Thus, the number of *hsp83* transheterozygotes is expected to be ~33%, unless the viability of the transheterozygous mutants is reduced.

TABLE 3

Male sterile mutation *scratch* fails to complement *hsp83* mutants

Mutant allele	Total progeny	*scratch*/mut	% of total	Germline
S38L (1D)	455	0	0	—
R48C (19F2)	1231	0	0	—
E317K (6D)	369	112	30	ms, fs
E377K (9J1)	443	0	0	—
S574C (3A)	439	146	33	ms, fs
S592F (6A)	667	35	5	ms, fs
S655F (4A)	658	162	25	ms, fs
P582	557	214	38	ms, ff
scratch	~1200	~600	~33	ms, ff

The transheterozygous progeny from a cross between two *hsp83* balanced stocks (*scratch* and a *hsp83* point mutation) were identified by the lack of dominant visible markers carried by the balancer chromosomes. Transheterozygous progeny were tested for fertility. Abbreviations are as defined in Table 1.

hsp83 and a primer specific for the *P*-element termini (Pend). For example, a primer located just upstream of the first exon (P1, Figure 1A) produced a product of ~450 bp when used with the primer Pend (Figure 1B, lane 2) and DNA from *scratch* flies. The genomic sequence of *hsp83* (Holmgren *et al.* 1979) predicts that the distance between primer P1 and the end of the first exon is 386 bp (Figure 1A). Given that the amplified product is >386 bp, the insertion point must be in the intron, ~60 bp from the junction of the first exon and the intron. Other experiments using additional PCR primers produced PCR fragments consistent with this conclusion (*e.g.*, the P2 and Pend primers produced a 2.2-kb fragment, Figure 1). No DNA fragment was amplified using the same primer sets when DNA from wild-type flies was used. Therefore, both genetic and molecular analyses demonstrate that *scratch* is an allele of *hsp83*. The phenotype of *scratch* is consistent with this conclusion. Homozygous *scratch* flies are viable and female fertile, but male sterile (Castrillon *et al.* 1993).

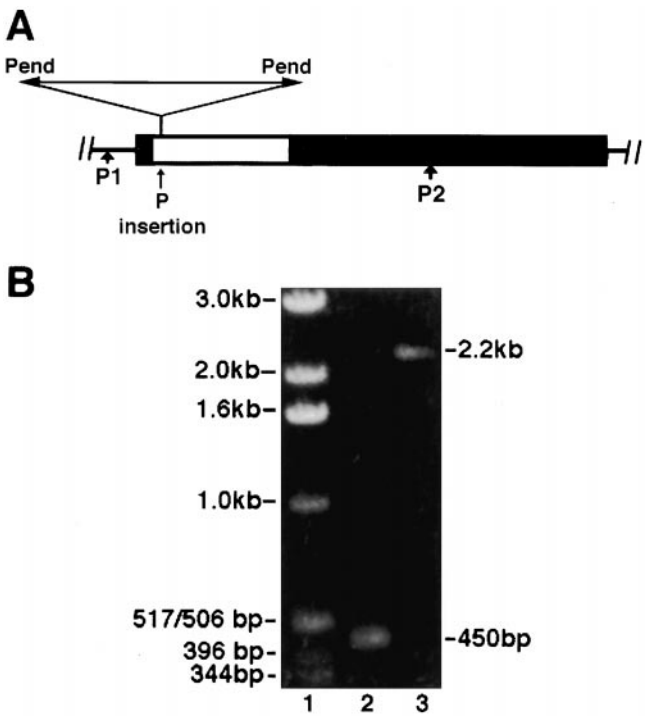

Figure 1.—The *P* element in *scratch* is inserted in the intron of the *hsp83* gene. (A) A schematic drawing of *hsp83* gene structure showing the two exons (black bars) and one intron (open bar). *hsp83*-specific primers P1 and P2 (sequences in materials and methods) were used together with a *P*-element-specific primer Pend to determine the *P*-element insertion site in *scratch*. The triangle represents the inserted *P* element and the arrows on the triangle represent the primer Pend, located at the *P*-element termini. (B) Representative results of PCR amplifications. Molecular size markers (lane 1); PCR products obtained by using primer P1 and Pend (lane 2, 450 bp) and the primers P2 and Pend (lane 3, 2.2 kb).

Hsp90 expression in Drosophila: To determine if the mutant phenotypes correlate with Hsp90 expression patterns, we analyzed Hsp90 levels during fly development using SDS-PAGE immunoblots and a monoclonal antibody specific for Drosophila Hsp90 (Figure 2). In wild-type flies Hsp90 levels remained essentially constant throughout the first 20 hr of embryogenesis (Figure 2A). Similarly, Hsp90 levels in whole animal tissue extracts during embryogenesis, larval, pupal, and adult stages were comparable (Figure 2B, lanes E, L, P, F, and M). Hsp90 levels were, however, increased in adult gonadal tissues (Figure 2B, compare lanes Fc, O, Mc, and T), particularly in males (Figure 2C). (Equal quantities of total protein were loaded on each lane.) This was particularly evident in males where testes (T) contained high levels of Hsp90 relative to male carcasses (Mc). As previously suggested (Kemphues *et al.* 1983), tubulin also exhibited a dramatic increase in expression in testes relative to carcasses (Figure 2B, Mc, and T). Hsp90 was somewhat enriched in ovaries (Figure 2B, Fc and O); tubulin was not (Figure 2B, Fc and O).

Reduced levels of Hsp90 protein lead to male sterility in *scratch*: Immunoblot analyses were performed to determine the effect of *scratch* on Hsp90 protein expression (Figure 3). Using phosphoimaging and radiola-

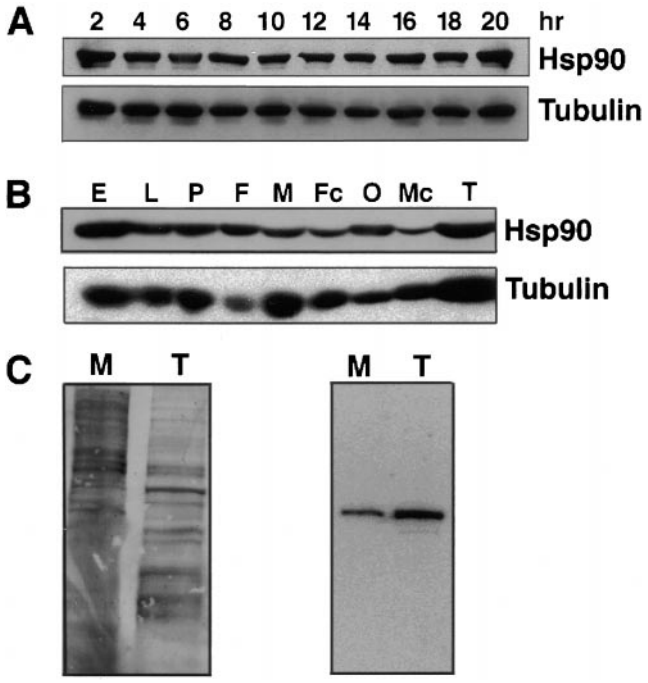

Figure 2.—Hsp90 is induced during germline development. Proteins, 10 μg per lane, were separated by SDS-PAGE and transferred to filters. (A) Immunodetection of Hsp90 (top) and tubulin (bottom) expression during embryogenesis. Fertilized eggs were collected and aged at 25° for the times shown prior to sample preparation. (B) Immunodetection of Hsp90 (top) and tubulin (bottom) protein expression during various stages of fly development. E, 0- to 18-hr embryos; L, larvae; P, pupae; F, whole females; M, whole males; Fc, female carcasses with ovaries removed; O, ovary; Mc, male carcasses with testes removed; T, testis. (C) Hsp90 protein levels in male tissues. Proteins of whole males (M) and testes (T) were separated by SDS-PAGE, transferred to a filter, and visualized by Coomassie blue staining (left) and reaction with anti-Hsp90 antibody (right).

beled ^{125}I-protein A to measure Hsp90 levels, the protein was reduced approximately threefold in ovaries, testes, and male bodies of homozygous *scratch* mutants. Immunoblots of two-dimensional gels using anti-Hsp90 antibody detected three major isoforms of Hsp90 present in testes, and *scratch* mutants contained all three (data not shown). Thus, *scratch* appears to cause a general decrease in Hsp90 levels rather than the loss of a particular isoform. As no reduction in female fertility was observed in *scratch* homozygotes, these data and the genetic analyses presented above suggest that spermatogenesis is particularly sensitive to reductions in Hsp90 function. Because the effects of *Hsp83* mutations on female fertility varied greatly between genotypes and male sterility was fully penetrant in all crosses, we concentrated further analysis on the process of spermatogenesis.

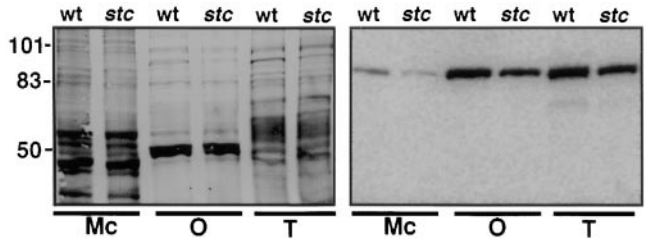

Figure 3.—Hsp90 protein production is reduced in *scratch* mutants. Proteins from wild-type (Wt) and homozygous *scratch* (*stc*) male carcasses, ovaries, and testes were separated by SDS-PAGE, transferred to filters, and visualized by Coomassie blue staining (left) and reaction with anti-Hsp90 antibody (right). Mc, male carcasses with testes removed; O, ovary; T, testis.

Hsp90 expression patterns during spermatogenesis: First, the cellular location of Hsp90 in wild-type flies was determined by confocal microscopy and indirect immunofluorescence using an anti-Hsp90 antibody, along with anti-DROP-1 antibody (a monoclonal antibody that recognizes both immature and mature sperm; Graner *et al.* 1994). In general, strong Hsp90 staining was detected throughout spermatogenesis. As expected from studies of Hsp90 localization in other cell types (Pratt 1992; Perdew *et al.* 1993; Ohsako *et al.* 1995; Perrot-Applanat *et al.* 1995; Biggiogera *et al.* 1996; Czar *et al.* 1996), Hsp90 staining was observed in the cytoplasm of spermatocytes (Figure 4, A and B). We also detected weak but reproducible staining in the nuclei of primary spermatocytes (Figure 4B, arrow) and staining of a mesh-like structure in the cortical regions of spermatocytes (Figure 4C). During late stages of sperm development, strong staining of spermatid bundles was observed (Figure 4D). Hsp90 immunostaining decreased significantly following sperm individualization (Figure 4F, arrow). Sporadic punctate staining of Hsp90 was observed along the length of the sperm tail in sperm bundles and individual sperm (Figure 4F, arrowhead). Thus, these data suggest that Hsp90 levels are highest in developing sperm and are dramatically reduced in mature sperm as cytoplasm is lost during individualization. These results are consistent with a role for Hsp90 during spermatogenesis.

Analysis of *hsp83* mutant phenotypes during spermatogenesis: Drosophila is an especially useful model system for the study of spermatogenesis because of its inherent spatio-temporal pattern of development (for reviews, see Lindsley and Tokuyasu 1980; Fuller 1993). Spermatogenesis requires coordination of several microtubule-mediated processes including mitosis, meiosis, the shaping of the sperm nucleus, elongation of the specialized mitochondrial derivative, and assembly of the motile sperm tail axoneme. When we examined spermatogenesis in viable *hsp83* mutants in detail, we found that all stages involving microtubule-mediated processes (Table 4, Figure 5) were affected.

During early stages of spermatogenesis, stem cells divide to produce a spermatogonial cell that undergoes four rounds of mitosis to produce 16 primary spermatocyte cyst cells (Fuller 1993). Phase-contrast microscopy

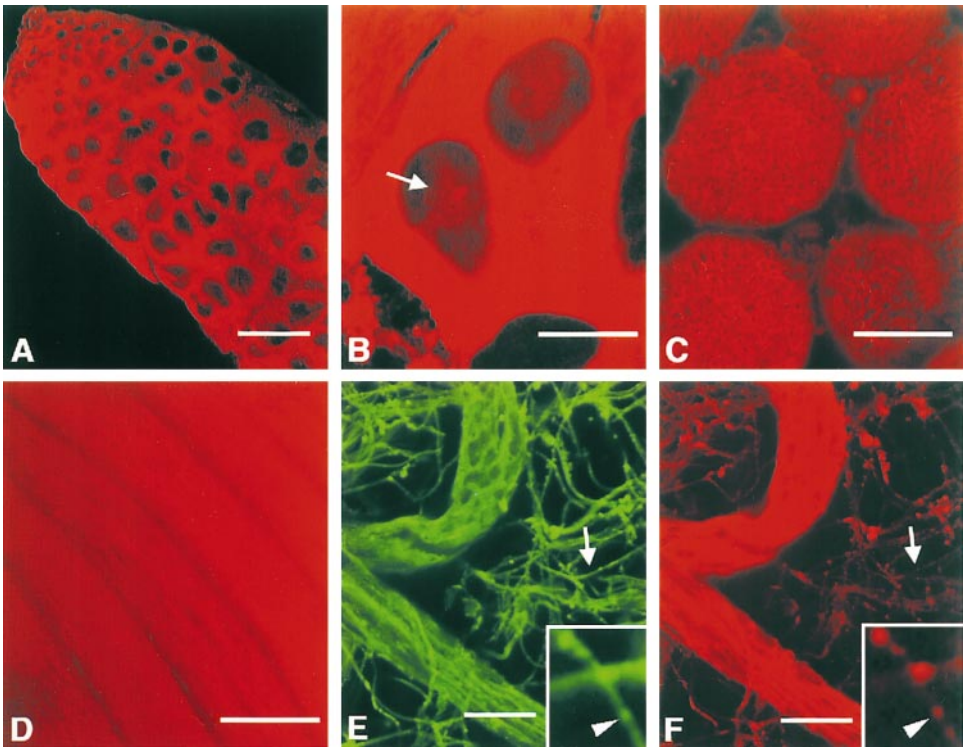

Figure 4.—Indirect immunofluorescence of Hsp90 and DROP-1 immunostaining in wild-type flies. Adult testes were dissected, fixed, and stained by indirect immunofluorescence using anti-Hsp90 or anti-DROP-1 antibody and Texas red- or FITC-labeled secondary antibody. (A) Low and (B and C) high magnifications of Hsp90 staining from a region near the apical end of a testis. (A and B) Generalized strong cytoplasmic staining (B, arrow) with weak nuclear staining in primary spermatocytes and (C) cortical fibrillar staining. (D) Hsp90 localization along developing spermatid bundles. Costaining with (E) anti-DROP-1 and (F) anti-Hsp90 antibodies demonstrates the relatively low level of Hsp90 staining in mature sperm (arrows) compared to the staining of immature sperm in the sperm bundle at the left. The insets show punctate staining in mature sperm tails (arrowheads). (A, D, E, and F) Bars, 50 μm. (B and C) Bars, 10 μm.

of squashes of mutant testes revealed severe abnormalities in the mitotic divisions that produce the 16 primary spermatocytes in 3 of the 12 mutants examined (Table 4). These mutants produced excessive numbers of primary spermatocytes (data not shown). In other transheterozygous male-sterile mutant combinations, most primary spermatocyte cysts appeared normal, but 5–10% showed abnormal morphologies, containing novel subcellular structures, variable numbers of cells (Figure 5, B and C), and/or large vacuolated cysts (Figure 5C). One other consistent abnormality observed was the presence of long, thin needle-like structures (Figure 5, B and C), which resemble those described in the mutant *Stellate* (Livak 1984; Pimpinelli *et al.* 1985; Palumbo *et al.* 1994; Bozzetti *et al.* 1995).

In the next stage of spermatogenesis, primary spermatocytes undergo meiosis, forming bundles of 64 spermatids, which then undergo nuclear maturation and axoneme elongation. During the early stages of this developmental period in wild-type flies, the paired nuclei and nebenkern (Figure 5D, light and dark bodies, respectively) are prominent (Fuller 1993). The nebenkern is a mitochondrial derivative produced as mitochondria fuse directly next to the nucleus. In 11 of the 13 mutant combinations analyzed, >90% of primary spermatocytes showed defects in meiosis (Figure 5, E and F; Table 4). Both nuclear and nebenkern morphologies were substantially altered in *hsp83* mutants (Figure 5, E and F). The size and shape of spermatids were abnormal and the number appeared to be reduced (data not shown). As previously observed in *scratch* mutants (Castrillon *et al.* 1993), optically refractile needle-like structures, similar in size, shape, and location to those observed in the primary spermatocytes, were also observed at this stage (Figure 5, B, C, E, and F). Multiple nuclei and multiple mitochondrial derivatives of abnormal shape and variable size were present in many individual spermatids (Figure 5, E and F).

In the final stages of wild-type development, spermatids, nuclei, axonemes, and mitochondrial derivatives synchronously elongate (Fuller 1993). Following this growth phase, spermatids individualize and move into the seminal vesicle (Figure 5I). *hsp83* mutants also exhibited abnormalities in these stages (Figure 5, H and J): both the synchrony and morphology of the sperm within a bundle were altered (Figure 5, G and H). This was particularly evident in mature sperm in the seminal vesicle where sperm nuclei were often kinked and shortened relative to the wild type (Figure 5, I and J). Individual sperm tails were fragile and broke more easily than wild-type sperm during dissections. Sperm motility was examined in mutant, transheterozygote, and wild-type crosses. No mutant crosses, except S574C/S38L, which had very weak motility, displayed evidence of the rapid sine wave motion typical of the transheterozygotes and wild type (data not shown).

Ultrastructural analysis of spermatogenesis in *scratch*: Defects in spermatogenesis were further analyzed by transmission electron microscopy. We focused on the *scratch* mutation because, as a *P*-element insertion in the intron, it results only in a reduction in wild-type Hsp90 function, eliminating the possibility that any defects

TABLE 4

Phenotypic analysis of spermatogenesis in *hsp90* mutants

Genotype	Defects[a]
E377K/S574C	Number, shape of spermatocytes within 16-cell cysts are mostly normal[b]. No needle-shaped crystals observed in developing spermatocytes, spermatids. Some motile sperm are present.[c]
E377K/S655F	Number, shape of spermatocytes within 16-cell cysts and nuclei, nebenkern within spermatids are mostly normal[b]. No needle-shaped crystals observed in developing spermatocytes, spermatids. Individualized sperm are present, but not motile.
stc/*stc* *stc*/P582 S38L/R48C E377K/S592F R48C/S574C S38L/E377K *stc*/S574C[e]	Number, shape of spermatocytes within 16-cell cysts are mostly normal[b]. Spermatids with variable number, size, shape of nuclei, nebenkern were observed throughout developing spermatids.[d] Needle-shaped crystals are present throughout developing spermatocytes, spermatids. Individualized sperm are present, but not motile and break easily during dissection.
stc/E317K *stc*/S592F E317K/E377K *stc*/S655F[g]	Excessive number of primary spermatocytes in many developing cysts.[f] Spermatids with variable number, size, shape of nuclei, nebenkern were observed throughout developing spermatids. Needle-shaped crystals are present throughout developing spermatocytes, spermatids. Individualized sperm are present, but not motile and break easily during squash.

[a] Listed in increasing severity of the phenotypic defects observed. Where more than one genotype is listed, except where noted by superscript, phenotypes observed were essentially identical.
[b] Abnormal structures observed were 5–10%.
[c] Although some motile sperm were observed, males were not fertile (data not shown).
[d] Variable numbers (20–70%) and degree of severity, observed both within and between different heteroallelic combinations.
[e] Crystal structures observed in some, but not all, stages of development in *scratch*/S574C.
[f] Greater than 90% abnormal.
[g] Phenotype of *scratch*/S655F identical to others in this group except no crystal structures were observed.

might be due to neomorphic effects of aberrant Hsp90 protein. A typical cross section of a wild-type sperm bundle shows elongating spermatids, each containing two mitochondrial derivatives and one axoneme with the expected 9+2 inner and outer microtubule elements (Figure 6, A and C). In *scratch* testes, bundles containing aberrant axonemes (double axonemes, no axoneme) and variable numbers of mitochondrial derivatives (1–10) were observed (Figure 6B). Empty spaces between elongating spermatids indicated that the packing and spacing of spermatid bundles were disrupted in *scratch* testes (Figure 6B). A double membrane structure normally encloses the axoneme (Figure 6C, arrow). However, *scratch* sperm axonemes often had missing or incomplete membranes (Figure 6D, arrow). Other abnormalities were noted, including stretches of what appeared to be outer doublet microtubules aligned along a double membranous structure (Figure 6D, large arrow) but not associated with a central pair, double membranous structures around incomplete axonemes (small arrow), and occasional individual outer doublet microtubules in the cytoplasm (arrowhead).

Biochemical analysis of Hsp90 interactions with purified microtubule proteins: The extensive developmental defects in sperm axonemes and associated structures in *hsp83* mutants suggested that Hsp90 might directly interact with microtubules in Drosophila, as it has been reported to do in other systems (Sanchez *et al.* 1988; Redmond *et al.* 1989). Microtubules were purified from embryonic extracts by taxol-induced assembly and analyzed on SDS-PAGE gels (Figure 7A). Total protein and protein present in the supernatant following assembly are shown in 7A, top, lanes 1 and 2, respectively. The microtubule pellet (Figure 7A, lane 3) was further purified by resuspension in buffer and centrifugation through a sucrose cushion (Figure 7A, lanes 4–7). Purification was evident from the prominent tubulin band migrating at 55 kD (Figure 7A, arrow). (This band is a minor component of total protein in the cell extract.) Levels of Hsp90 were high in the cell extract, supernatant, and first pellet (Figure 7A, bottom, lanes 1–3), decreased significantly following centrifugation through sucrose, and then remained constant for each successive wash and centrifugation (Figure 7A, bottom, lanes 4–7). Thus, only a small fraction of Hsp90 copurified with the microtubule pellet.

To further probe the nature of Hsp90/microtubule interactions, microtubule proteins were purified by repeated cycles of temperature-induced assembly and disassembly (Figure 7B). This method of purification differs from the taxol-induced method in that each cycle represents the *de novo* assembly of microtubules. Again, microtubule purification was apparent from the increasing abundance of tubulin (Figure 7B, arrow) through

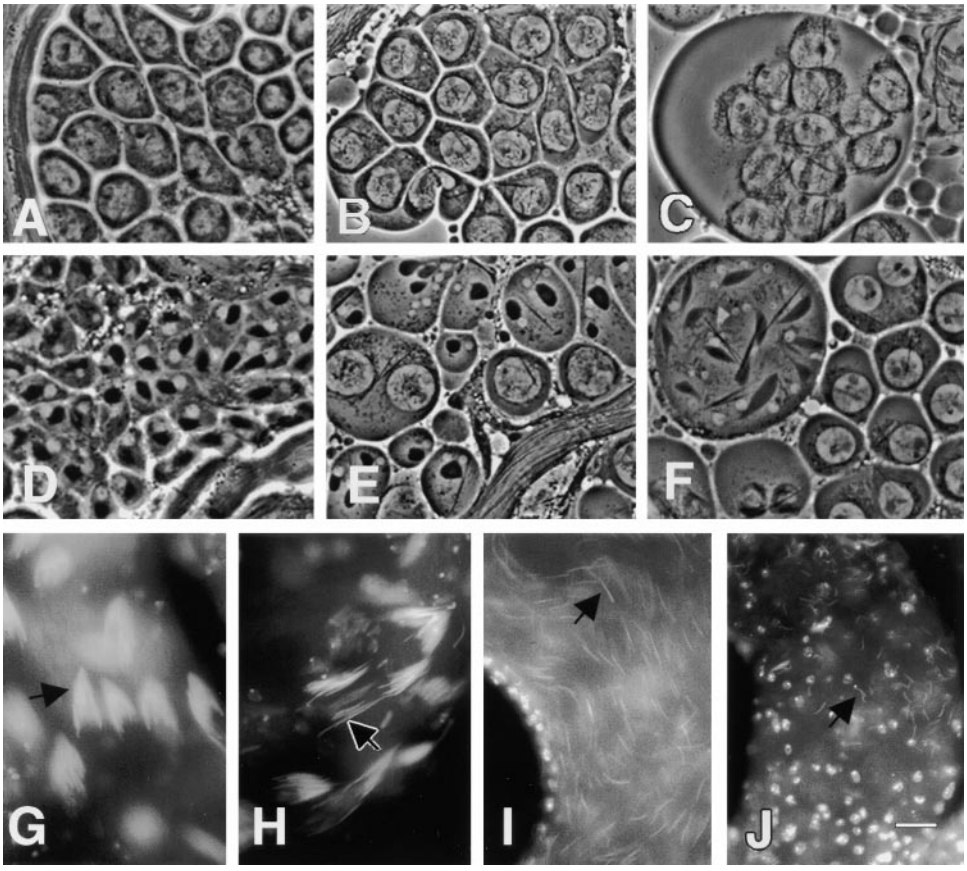

Figure 5.—Spermatogenesis defects in *hsp83* mutants. Squash preparations of wild-type and *hsp83* mutant testes. (A) Wild-type primary spermatocytes displaying typical organization, with regular packing and spacing of cells. (B) Primary spermatocytes from transheterozygous (P582/*scratch*) flies and (C) transheterozygous (S574C/R48C) flies, exhibiting needle-like structures and aberrant cell packing. (D) Wild-type onion stage cells following meiosis II, showing condensed nuclei and nebenkerne. (E and F) Aberrant primary spermatocytes and early onion stages in the transheterozygous mutant S574C/R48C with prominent needle-like structures clearly visible. (G–J) Sperm bundles in testes and sperm in seminal vesicles. Testes, including seminal vesicles, were dissected, fixed, and stained with a DNA-specific fluorochrome and visualized by fluorescence. (G) Wild-type and (H) S38L/R48C sperm bundles showing cysts of elongating sperm nuclei (arrows) prior to individualization. (I) Wild-type and (J) S574C/R48C sperm in seminal vesicles. Arrows point to highly fluorescent sperm heads. (J) Bar, 10 μm. All panels are at the same scale.

each round of assembly and disassembly. (The identity of this band as tubulin was confirmed by immunoreactivity with an antitubulin monoclonal antibody, data not shown.) Hsp90 and the constitutive relatives of Hsp70 (Hsc70) were monitored in these same fractions (Figure 7B, bottom panels). TBP was included as a control for nonspecific binding. Both Hsp90 and Hsc70 copurified with microtubules through the first two rounds of purification (Figure 7B, Hsp90 and Hsp70, lanes 2–5), were weakly represented in the third (Figure 7B, Hsp90 and Hsp70, lanes 2–5), and were undetectable in the fourth (Figure 7B, Hsp90 and Hsp70, lanes 2–5). TBP showed the same binding profile as Hsp90 and Hsc70. Thus, no significant binding of Hsp90 to microtubules was detected under these experimental conditions.

Microtubules are aberrantly distributed in yeast cells expressing mutant Hsp90 proteins: Previously, we described the isolation of several Hsp90 temperature-sensitive point mutations in *S. cerevisiae*. Most of these have reduced function at all temperatures and are temperature-sensitive for growth simply because they cannot satisfy the increased demand for Hsp90 function at higher temperatures (Nathan and Lindquist 1995). One, however, behaves as a classic temperature-sensitive mutant: *Hsp90*^{G170D} has wild-type activity at 25° and very rapidly loses activity upon a shift to high temperature. This loss of activity is due to a structurally destabilizing glycine-to-aspartic-acid substitution in the hydrophobic core of the N-terminal domain (Prodromou *et al.* 1997a,b; Stebbins *et al.* 1997). To determine whether Hsp90's role in microtubule dynamics is generally conserved, we examined tubulin distributions after incubation at the nonpermissive temperature.

Diploid yeast cells expressing either wild-type Hsp90 or Hsp90^{G170D} were grown to mid-log phase at 25° and then incubated at permissive (25°) or nonpermissive (37°) temperature for 3 hr. At 25° wild-type and Hsp 90^{G170D} cells had nearly identical, normal tubulin distributions (Figure 8, top row). At 37° wild-type cells displayed similar structures with a somewhat different distribution, consistent with the transient, partial block in the cell cycle that occurs with such temperature shifts in yeast (Figure 8, bottom left). In marked contrast, Hsp90^{G170D} cells exhibited a grossly altered tubulin distribution after incubation at 37°, specifically, a remarkable proliferation of cytoplasmic microtubules (Figure 8, bottom right). This increase in cytoplasmic microtubules was not specific to this mutation. It was seen in strains expressing other mutant hsp90 proteins and in strains expressing very low levels of wild-type Hsp90. Furthermore, it was observed before cells lost viability (data not shown). Thus, Hsp90 plays a crucial role in tubulin

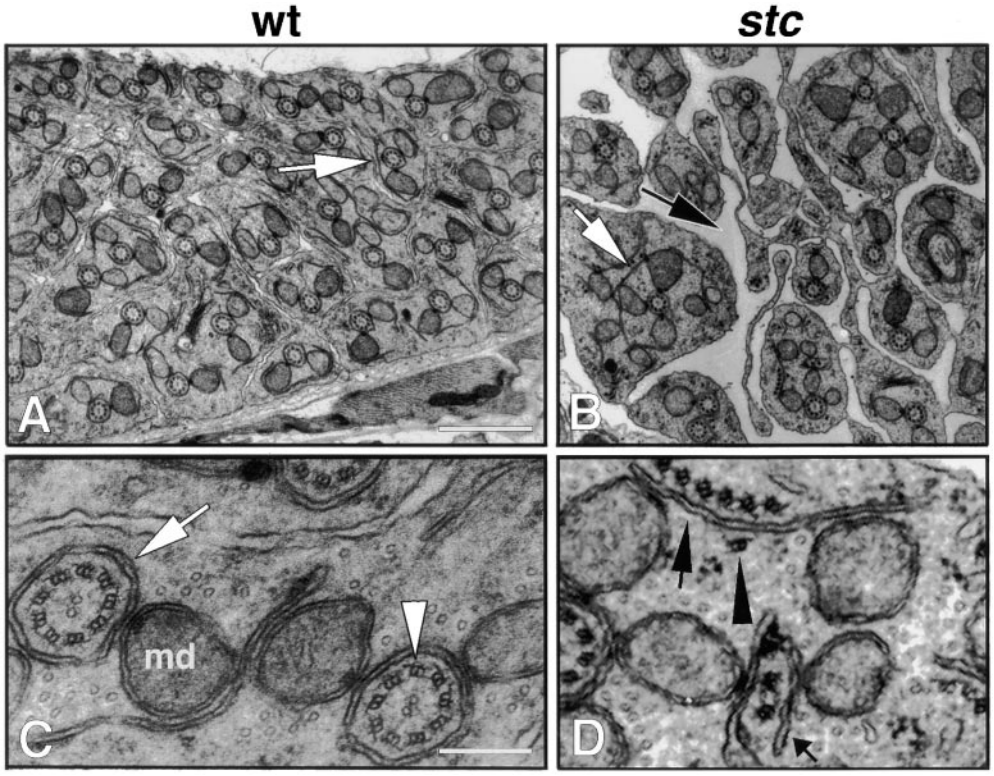

Figure 6.—Transmission EM analysis of the *scratch* mutant phenotype. Developing spermatids of (A) wild-type and (B) *scratch* testes. (A, arrow) Individual wild-type spermatids are close packed and contain a single axoneme and paired mitochondrial derivatives enclosed by a membrane. (B, black arrow) *scratch* spermatids are loosely packed and contain (B, white arrow) various numbers of axonemes and mitochondrial derivatives. (A) Bar, 1 μm. A and B are at the same scale. (C) At higher magnification, wild-type axonemes contain 9+2 microtubule arrays (the arrowhead points to one microtubule outer doublet) closely opposed to paired mitochondrial derivatives (md) surrounded by a double membrane (arrow). Numerous defects in axoneme organization are observed in *scratch* spermatids including (D, large arrow) incomplete membranes and linear arrays of outer doublet microtubules and (D, arrowhead) occasional solitary outer doublet microtubules. (C) Bar, 0.2 μm. C and D are at the same scale.

dynamics in at least two very different eukaryotic organisms.

DISCUSSION

A function for Hsp90 in spermatogenesis: The essential and central roles of Hsp's in cellular metabolism and regulation have made it very difficult to obtain the viable alleles needed to establish the most critical functions of any Hsp's in the developmental processes of metazoans. Here, we have solved this problem for the Hsp90 protein of Drosophila by analyzing two classes of mutations. First, we found that several previously identified point mutations that are lethal as homozygotes (Cutforth and Rubin 1994; van der Straten *et al.* 1997) are viable as transheterozygotes. Second, we discovered that a previously studied homozygous viable mutant, *scratch*, is an allele of *hsp83*, with a *P*-element insertion in the intron. The majority of adult flies carrying viable *hsp83* mutations display no overt developmental defects. Indeed, most show full viability, emerging in crosses at normal ratios. They also exhibit no overt changes in developmental timing or behavior in the embryonic, larval, or pupal stages. All of the flies, however, are male sterile, whether derived from the transheterozygous combination of point mutations, or from the homozygous *P*-element insertion. This establishes spermatogenesis as the single most sensitive developmental process to Hsp90 function in Drosophila.

Spermatogenesis in Drosophila has been the subject of intense investigation over the past three decades (reviewed in Lifschytz 1987; Fuller 1993). Male-sterile mutants are common: both classical genetic analyses and *P*-element mutagenesis suggest male sterile mutants represent ~10–15% of the total number of recessive lethal mutations (Wilkinson *et al.* 1974; Lifschytz 1978; Cooley *et al.* 1988). In most cases the first observed deficits in spermatogenesis were seen in primary spermatocytes, resulting in either the complete absence of mature sperm or the presence of highly disorganized, nonmotile sperm (Fuller 1993). To date, only four male-sterile mutants have been isolated and analyzed that produce mature, motile sperm. These are capable of egg penetration, but do not initiate egg development (Baker 1975; Fuyama 1984, 1986; Yasuda *et al.* 1995), defining a class of paternal-effect lethal genes. The *Hsp83* mutant phenotypes we have observed are unusual and fall in between these two extremes: spermatogenic de-

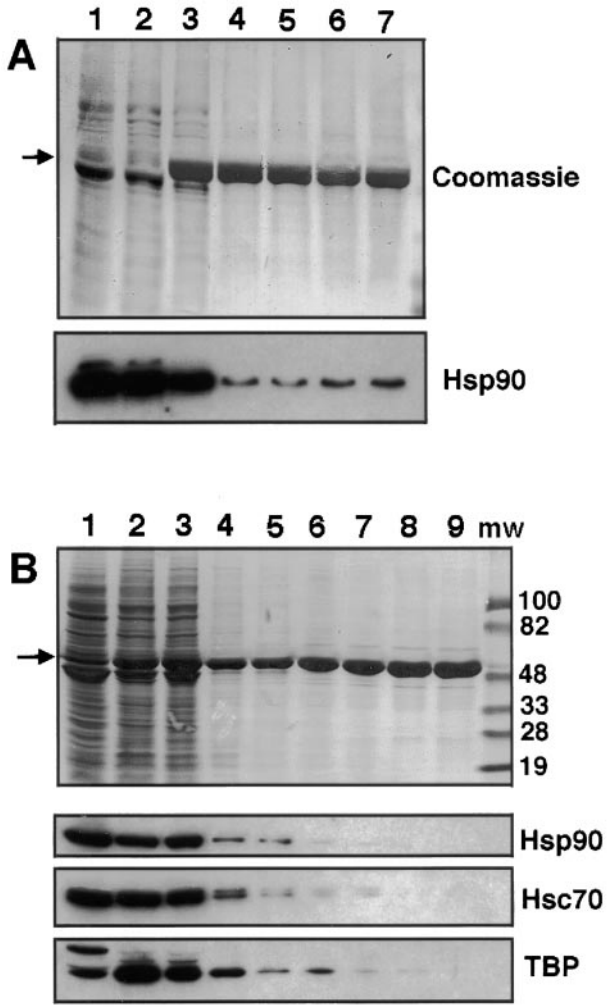

Figure 7.—Association of Hsp90 with purified microtubules from embryo extracts. Microtubule proteins were purified from embryonic extracts by (A) taxol-induced microtubule assembly and (B) temperature-induced cycles of assembly and disassembly and separated by SDS-PAGE. Top panels, Coomassie blue staining; bottom panels: (A) immunological detection of Hsp90 and (B) bottom panels, Hsp90, Hsc70, and TATA-binding proteins. Each lane contained 10 μg of protein. (A) lane 1, cell extract; lane 2, supernatant from first centrifugation; lanes 3–7, pellets from the first through fifth rounds of centrifugation. (B) lane 1, cell extract; lanes 2, 4, 6, and 8, pellets from the first, second, third, and fourth rounds of temperature-induced assembly; lanes 3, 5, 7, and 9, supernatants following the first, second, third, and fourth rounds of cold-induced disassembly; last lane, molecular weight markers.

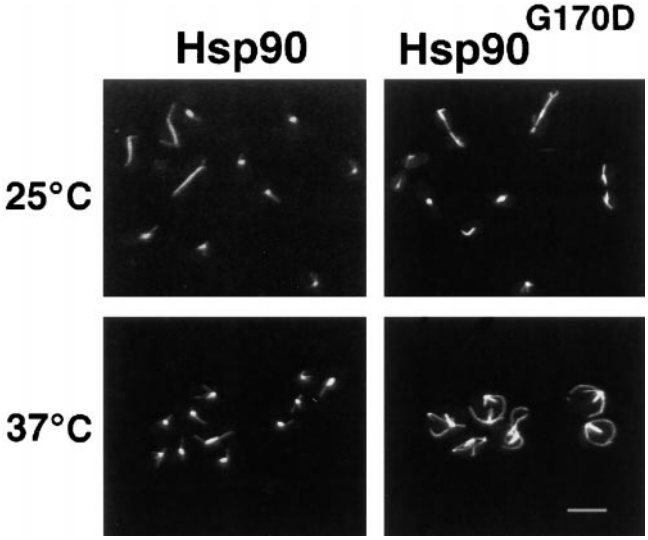

Figure 8.—Effect of Hsp90 mutations on microtubule structures in yeast. Log-phase diploid yeast cells expressing either wild-type Hsp90 or the Hsp90^{G170D} mutant were incubated for 3 hr at either the permissive temperature (25°) or the nonpermissive temperature (37°) and stained with anti-tubulin antibodies. The normal distribution of tubulin-staining patterns, characteristic of different stages in the cell cycle, is seen in wild-type cells at both temperatures and in Hsp90^{G170D} cells at 25°. At 37°, Hsp90^{G170D} cells contain extensive, abnormal arrays of cytoplasmic microtubules. Bar, 10 μm.

fects are observed through all stages of spermatogenesis and yet, in one allelic combination, some motile sperm are produced, but these do not fertilize eggs (T. L. Karr, L. Yue and S. Rutherford, unpublished results).

Complementation patterns: The inviability of all Drosophila point mutants as homozygotes and the viability of several in transheterozygous combinations has two possible explanations. The first follows from the observation that Hsp90 interacts with its substrates in a dynamic pathway that includes several other cochaperones that join and leave at various times (Smith 1993). If complementing alleles of *Hsp83* have defects at different steps and if substrates can shuttle between complexes with different defects, they could be shepherded through the entire pathway. The second explanation derives from previous genetic and biochemical data indicating that Hsp90 functions as a dimer (Minami *et al.* 1994). Proteins containing different individual defects might produce a mixed dimer that is capable of guiding substrates through the entire pathway. Of course these observations are not mutually exclusive, but, in either case, one might expect that complementing alleles would have the tendency to map to different domains.

The domain structure of Hsp90 has been defined, in part, through the isolation of regions stable to proteolysis. The crystal structure of the N-terminal domain has been solved for the bovine and yeast proteins and corresponds to approximately the first 225 amino acids on the Drosophila sequence. Three α-helices and three β-sheet strands in this domain form a deep pocket that can bind both geldanamycin and ATP (Prodromou *et al.* 1997a,b; Stebbins *et al.* 1997). This domain is followed by a middle domain (Stebbins *et al.* 1997), corresponding approximately to amino acids 271–587 in the Drosophila sequence. The C-terminal region contains a dimerization activity (Minami *et al.* 1994; Wearsch and Nicchitta 1996) and corresponds to Drosophila residues 596–677.

The mutants we analyzed (Cutforth and Rubin 1994; van der Straten *et al.* 1997) map to each of the three domains: the N-terminal region (S38L, R48C),

the middle region (E317K, E377K, S574C), or the C-terminal region (S592F, S655F). The complementation patterns we observe are in part consistent with current information on Hsp90 domain structure, but also provide some puzzles. For example, none of the C-terminal mutants complement each other (Table 1), and the one that maps closest to the dimerization region, S655F (Minami *et al.* 1994), fails to complement most of the other mutants. This is consistent with the notion that proteins with similar defects cannot compensate for each other, and that dimerization is important for Hsp90 function. Curiously, however, two mutations located in the middle domain, E317K and E377K, do complement each other. Underscoring the unexpected nature of this complementation, E317K is one of the strongest alleles and complements no other point mutation. A similar puzzle is that the two mutations in the N-terminal domain, S38L and R48C, complement each other. Remarkably, in the crystal structure of this domain, S38L and R48C map to the same helix, which lines the ATP/geldanamycin binding pocket (Prodromou *et al.* 1997a,b; Stebbins *et al.* 1997). These complementation patterns represent fascinating subjects for further structure/function analysis of Hsp90.

Phenotypic specificity of Hsp90 mutants: Because the mutations we employed are located in different regions of the protein, it is particularly striking that every viable transheterozygote exhibits the same phenotype. Moreover, this same phenotype occurs in flies homozygous for a *P*-element insertion in the *hsp83* intron that simply reduces Hsp90 expression. Thus, male sterility is due to a reduction in Hsp90 function rather than to possible neomorphic effects of the point mutations. The effects of Hsp90 mutations on female fertility are less severe. Most viable transheterozygous females produce progeny, albeit generally at less than 20% the rates of their heterozygous wild-type siblings (Table 2, and data not shown). Furthermore, although *scratch* homozygotes are 100% penetrant for male sterility, they exhibit no overt defects in female fertility.

Cytological and ultrastructural examination suggests it is the microtubule-based processes in spermatogenesis that are most affected in *scratch*. None of the defects is fully penetrant. Rather, groups of developing cells are affected at different stages. Defects begin with the first mitotic divisions, where coordination of the stereotypic and highly programmed pattern of sperm development is lost. Meiotic divisions are also abnormal and most sperm are not properly formed. The sperm tail is primarily composed of an axoneme containing microtubules and associated proteins, and paired mitochondrial derivatives. The basic components of the 9+2 axonemal structure are present (*i.e.*, the a and b subfibrils and central pair microtubules), but in many cases they are not properly configured in a functional structure. Finally, although some apparently mature sperm are produced and transferred to seminal vesicles, they are immobile and extremely fragile—fragmenting during dissection and mounting. These observations suggest that Hsp90 function is also necessary for establishing the structural stability of the axoneme. Of course, it is entirely possible that all of these defects are a consequence of just one early defect. However, because some of the transheterozygous mutant combinations exhibited no overt defects in early processes, it seems more likely that Hsp90 functions at several steps in sperm maturation.

The nature of the defects we observed suggest a role for Hsp90 in microtubule dynamics. However, microtubule function is essential for all cell divisions. Why, then, would flies defective in Hsp90 exhibit male sterility, only partial female infertility, and appear generally otherwise normal? Sperm have evolved for motility and mass production. Their development is therefore dependent on the rapid and highly coordinated execution of several different microtubule-based processes, including the synchronous mitotic divisions of primary spermatocytes, two meiotic divisions, complex changes in cell shape, and the structure of the sperm tail itself (Fuller 1993). Moreover, Drosophila produce unusually elongated sperm that contain concomitantly more microtubules than other organisms (Fuller 1993; Pitnick *et al.* 1995; Karr 1996). Thus, we suggest that the extreme sensitivity of spermatogenesis to Hsp90 defects in Drosophila points to a more general function for Hsp90 in microtubule-based processes. When Hsp90 function is compromised, spermatogenesis is the first developmental process overtly affected.

Consistent with this supposition, we find that abnormal microtubule structures are among the first detectable defects when temperature-sensitive yeast *hsp82* mutants are transferred to nonpermissive temperatures. These defects appear well before cells lose viability. That is, the cells retain the ability to form colonies upon return to normal temperatures. Thus, the microtubule defect is not a secondary effect on fitness. Again, these observations point to a role for Hsp90 in microtubule dynamics and further indicate this role is highly conserved.

The possible role of Hsp90 in microtubule functions: Immunofluorescent localization of Hsp90 in rat endothelial and PtK1 epithelial cells has led to the suggestion that most of the protein is associated with microtubules (Redmond *et al.* 1989). However, this observation has not been generally confirmed (Fostinis *et al.* 1992; Biggiogera *et al.* 1996). In yeast, Hsp90 shows a general and diffuse cytoplasmic localization with no apparent association with microtubules (L. Arwood and S. Lindquist, unpublished results). Similarly, our staining of Drosophila testes with Hsp90 antibodies did not suggest a particular association with microtubules. Only a very small portion of Drosophila Hsp90 cofractionated with taxol-stabilized microtubules. And negligible levels of Hsp90 were bound to microtubules purified through

repeated rounds of assembly and disassembly. Thus, both the cellular pattern of Hsp90 distribution and the biochemical evidence suggest that the majority of Hsp90 protein does not stably interact with microtubules in Drosophila.

Previous biochemical investigations in mammalian cells and genetic investigations in yeast have demonstrated that Hsp90 plays a key role in maintaining the activity of a wide variety of signal transducers, including both serine/threonine and tyrosine kinases (Smith 1995; Nathan *et al.* 1997; Pratt and Toft 1997). We suggest that the effects of Hsp90 on spermatogenesis in general, and microtubule dynamics in particular, are indirect and indicative of a role for HSP90 in maintaining the function and stability of the signal transducers that control spermatogenesis. These are still largely undefined. However, genetic studies of the *Stellate* locus (Livak 1984; Pimpinelli *et al.* 1985; Palumbo *et al.* 1994; Bozzetti *et al.* 1995) suggest that CKII may be among them.

The intriguing needle-like structures within the cytoplasms of primary spermatocytes and developing spermatids of *hsp83* mutants are strikingly similar to those observed in the spermatocytes of XO males and of flies carrying deficiencies of locus *Yh11* on the Y chromosome, where *Suppressor of Stellate* resides (Livak 1984; Pimpinelli *et al.* 1985; Palumbo *et al.* 1994; Bozzetti *et al.* 1995). These crystals are proteinaceous and are caused by deregulation of the Stellate locus, which encodes multiple copies of the β-subunit-like proteins of CKII (Livak 1984; Pimpinelli *et al.* 1985; Palumbo *et al.* 1994; Bozzetti *et al.* 1995). The catalytic α-subunit of CKII is stabilized by Hsp90 and is extremely sensitive to changes in Hsp90 concentration (Miyata and Yahara 1992, 1995). Thus, it seems likely that reduced Hsp90 function in *hsp83* and *scratch* mutants leads to the formation of crystals because it alters the functional state of CKII and causes derepression of the β-like loci on the Y chromosome. These observations provide an immediate avenue for investigating how changes in Hsp90 function alter the activity of a particular target protein and its consequences in developmental processes. It should be noted, however, that some *hsp83* heteroallelic combinations produce male sterility without detectable Stellate crystals. Whether these genotypes affect CKII, but to a lesser extent, and how many other Hsp90 target proteins are affected remains to be determined.

The male sterility of Hsp90 alleles should provide a potent mechanism for identifying the specific factors that are most sensitive to the loss of Hsp90 function through genetic selection for the restoration of fertility. The Drosophila testis with its unique, readily visualized spatio-temporal pattern of development provides an excellent subject for defining their roles.

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