The consequences of expressing hsp70 in *Drosophila* cells at normal temperatures

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In *Drosophila* cells, regulatory mechanisms not only act to provide rapid induction of hsp70 during heat shock but also to prevent expression at normal temperatures. To determine whether expression of hsp70 is detrimental to cells growing at normal temperatures, we used heterologous promoters to force expression of the protein in tissue culture cells and in larval salivary glands. Initially, constitutive expression of hsp70 substantially reduces the rate of cell growth. With continued expression, however, growth rates recover. At the same time, the intracellular distribution of hsp70 changes. Immediately after induction, the protein is diffusely distributed throughout the cell, but as growth resumes it coalesces into discrete points of high concentration, which we term hsp70 granules. hsp70 granules are also observed both in wild-type *Drosophila* tissue culture cells and in salivary glands after extended periods of recovery from heat shock. The protein in these granules appears to be irreversibly inactivated. It cannot be dispersed with a second heat shock, and cells containing these granules do not show thermotolerance. Only partial overlap between hsp70 granules and lysosomes indicates that the granules form independently of lysosomes. We conclude that expression of hsp70 is detrimental to growth at normal temperatures. We suggest that the change in hsp70 distribution, from diffuse to granular, represents a mechanism for controlling the protein's activity by sequestration.

[*Key Words*: *Drosophila*; hsp70; regulation; protein sequestration]

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The heat shock response of *Drosophila* cells is one of the most intense inductions of gene expression known. At normal temperatures (25°C) heat shock protein 70 (hsp70) is virtually undetectable (Velazquez et al. 1983). Within minutes of a shift to 37°C, hsp70 becomes the major product of protein synthesis, and within 2 hr, it accumulates to become one of the most abundant proteins in the cell (Lindquist 1980a,b).

This remarkable induction is achieved by mechanisms acting at several different levels. First, multiple copies of the *hsp70* gene (five per haploid genome) are preassembled into an open chromatin configuration at normal temperatures (Wu 1980). RNA polymerase II is associated with these genes and appears transcriptionally engaged but arrested in elongation (Rougvie and Lis 1988). Upon shift to high temperatures, a transcription factor specific for heat shock genes (HSF) is activated, and the block in elongation is relieved (Parker and Topol 1984; Sorger and Pelham 1987; Zimarino and Wu 1987; O'Brien and Lis 1991). The absence of intervening sequences in the *hsp70* gene, a unique attribute of the heat-inducible members of the gene family, allows hsp70 transcripts to circumvent the block in intron processing that occurs at high temperatures (Yost and Lindquist 1986). Transcripts of the *hsp70* gene appear in the cytoplasm within minutes of exposure to elevated temperatures.

Two additional mechanisms for regulating hsp70 expression act in the cytoplasmic compartment. First, at elevated temperatures, the translation of normal cellular messages is strongly inhibited. Special sequences in the 5′ leaders of *hsp70* messages allow their rapid translation under these conditions (Klemenz et al. 1985; McGarry and Lindquist 1985). Second, at normal temperatures, sequences at the 3′ end of the *hsp70* message target it for degradation at an extremely rapid rate, eliminating any transcripts that escape normal transcriptional repression. Upon shift to high temperatures, degradation is immediately halted, allowing *hsp70* mRNA to accumulate to 10,000 copies per cell within 2 hr of heat shock (Lindquist 1980b; Petersen and Lindquist 1989).

The nature of these regulatory mechanisms suggests that minimizing the expression of hsp70 at normal temperatures may be as important as maximizing its expression at high temperatures. To examine this issue, we forced expression of the protein at normal temperatures by placing its coding sequence under the control of heterologous promoters. We find that expression of the protein at normal temperatures is deleterious. Cells do not die, but growth rates decrease sharply. Rapid growth resumes only as hsp70 coalesces into discrete locations within the cell. These results suggest that the activity of

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hsp70 at normal temperatures is repressed by its sequestration from other cellular proteins. Presumably, sequestration helps the cell to balance the beneficial effects of the protein during exposure to stress with its deleterious effects during normal growth.

Results

Production of cells that express hsp70 *constitutively*

In previous work we have transformed *Drosophila* tissue culture cells with many variant *hsp70* genes, including constructs that carry *hsp70* regulatory sequences joined in various combinations to *hsp70* deletion mutations, to antisense coding sequences, or to coding sequences from other genes (McGarry and Lindquist 1985, 1986; Petersen and Lindquist 1988, 1989; Yost and Lindquist 1988). The success rate for individual transfections in these experiments was generally >90%. However, attempts to transform cells with constructs that would provide increased expression of wild-type *hsp70* at normal temperatures failed repeatedly.

In the simplest case, we transfected cells with extra copies of the wild-type *hsp70* gene. In five separate experiments, each involving many independent transfections, most cultures failed to produce transformants. A few yielded resistant cells, but with a single exception, these showed no overexpression of hsp70. The line that did produce greater quantities of hsp70 upon heat shock also showed higher constitutive levels of expression. Although the level of constitutive expression was nearly 100-fold less than the level observed in wild-type cells after heat shock (Solomon et al. 1991), this line was unstable. In the same experiments, stable transformants appeared in every culture when the transfected *hsp70* gene carried a large deletion in the coding sequence (McGarry 1986; Rossi 1987).

To assay the expression of the transfected genes in such experiments, we then employed an *hsp70* variant that encoded a short extension at its carboxyl terminus recognized by a specific antibody (a construct employed previously in transient transfections with mammalian cells; Munro and Pelham 1984). As with the wild-type gene, it was difficult to obtain stable transformants with the tagged *hsp70* gene. Eventually, two cultures yielded resistant lines, but in both cases expression of the tagged protein was low. Again, parallel transfections with a variant carrying a deletion in the coding sequence readily yielded lines that expressed the transfected gene at a high level (Fig. 1A).

One explanation for these observations is that hsp70 is deleterious at normal temperatures, and only cells that happen to incorporate few copies of the construct, or many copies in a less active state, can survive. Alternatively, because hsp70 may repress its own synthesis (DiDomenico et al. 1982; Tilly et al. 1983; Stone and Craig 1990), a slight excess of hsp70 might prevent greater expression by down-regulating transcription from the hsp70 promoter. To distinguish between these possibilities, we forced expression of hsp70 at normal temperatures by use of heterologous promoters. In this and all subsequent experiments we employed wild-type *hsp70*-coding sequences to preclude the possibility that the peptide tag itself might have an adverse effect.

The first promoter employed was the constitutive *Drosophila* promoter *Eip28/29* (Cherbas et al. 1986). Again, most transformation attempts failed. In one case, after a very long period of selection, a transformant did appear (*eip300*). Although rates of hsp70 synthesis were low, because the protein is very stable and the cell cycle long (>36 hr), hsp70 accumulated to a substantial level. During growth at 25°C, accumulation was comparable to that of wild-type cells during the first 45 min of exposure

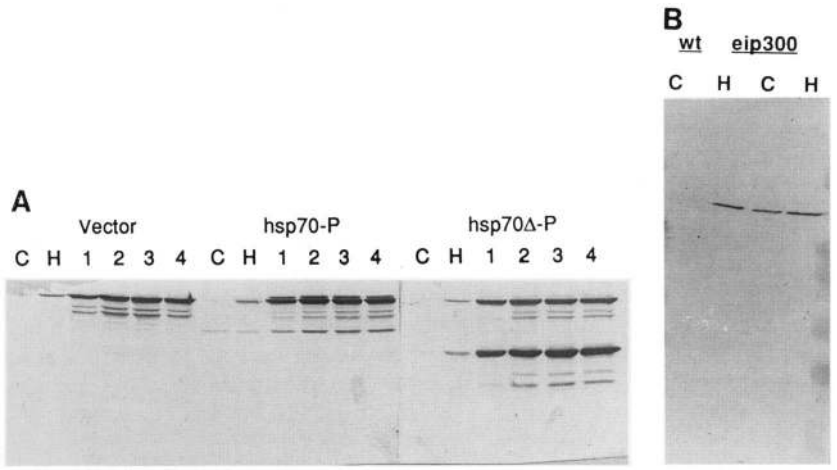

Figure 1. Levels of hsp70 expression in transformed cell lines. (*A*) Cells were transformed with the vector alone or with *hsp70* genes marked at their carboxyl termini by a small insertion encoding the substance P epitope (hsp70-P, the full-length *hsp70* gene; hsp70Δ-P, an *hsp70* gene carrying a deletion of the sequence encoding amino acids 115–338). Cells were maintained at 25°C (*C*) or shifted to 36.5°C for 45 min (*H*) and returned to 25°C for recovery for 1–4 hr (*1–4*). Electrophoretically separated proteins were transferred to nitrocellulose, reacted with monoclonal antibody 7FB (specific for hsp70), and visualized with horseradish peroxidase secondary antibody. The endogenous hsp70 protein and minor degradation products are the only bands visible in cells transformed with the vector alone. The wild-type protein tagged with substance P is visible just above the endogenous protein, as expected from its small increase in size. The tagged deletion protein is the prominent lower-molecular-mass species in the *right* panel. The positions of the substance P-tagged proteins were confirmed by staining duplicate blots with an antibody specific for substance P (data not shown). (*B*) Cells were transformed with the vector alone (wt) or with an *hsp70* gene placed under the control of the constitutive promoter from the *Eip28* gene (cell line *eip300*). Cells were maintained at 25°C (*C*) or heat-shocked at 36.5°C for 45 min (*H*). *hsp70* was visualized as in *A*, but with the staining allowed to proceed further.

to 36.5°C, but severalfold less than the level achieved during recovery (cf. lane C, for the *eip300* line in Fig. 1B, with lanes H in Fig. 1A and B).

Eip300 cells had high levels of viability (by trypan blue staining), but they grew very slowly. Nevertheless, they responded to heat shock and recovered from heat shock with the same kinetics as control cells transformed with the selectable marker alone (Fig. 2). This was surprising because previous studies have suggested that hsp70 plays a role in repressing the expression of itself and of other heat shock proteins during recovery (DiDomenico et al. 1982; Tilly et al. 1983; Stone and Craig 1990). The absence of an effect on regulation prompted us to investigate the subcellular distribution of hsp70 in *eip300* cells.

Intracellular distribution of constitutively synthesized hsp70

When *eip300* cells were stained with a monoclonal antibody specific for hsp70, staining was heterogeneous

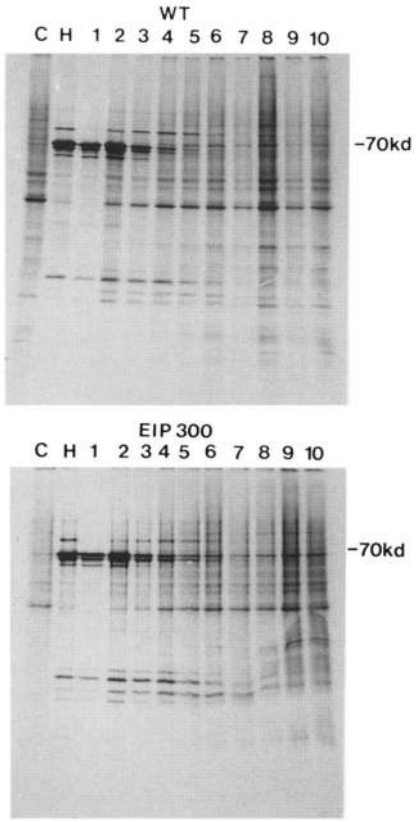

Figure 2. Protein synthesis during heat shock and recovery in cells that express hsp70 constitutively. Wild-type cells transformed with the antibiotic-resistance vector alone (WT) and cells transformed with *hsp70*-coding sequences under the control of the constitutive *eip28* promoter (*eip300*) were maintained at 25°C (*C*) or heat-shocked at 36.5°C for 45 min (*H*) and returned to 25°C for recovery (*1–10*). Cells were pulse-labeled with [^{3}H]leucine at 25°C, at 36.5°C, or at hourly intervals during recovery up to 10 hr. Electrophoretically separated proteins were visualized by fluorography.

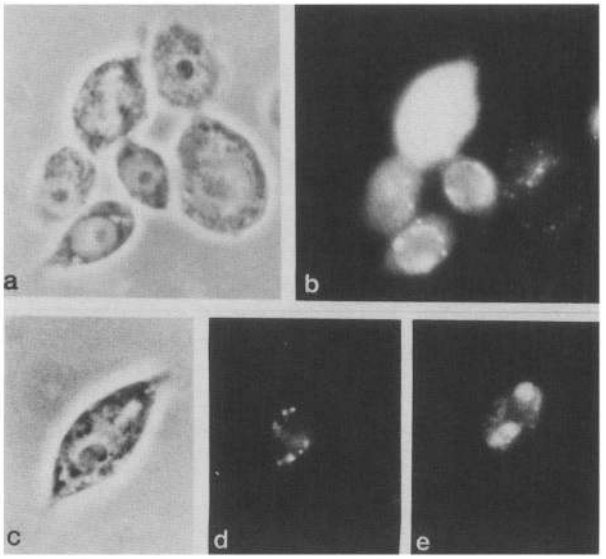

Figure 3. Immunofluorescent localization of constitutively expressed hsp70. Tissue culture cells transformed with *hsp70*-coding sequences under the control of the constitutive *eip28* promoter (line *eip300*) were reacted with a monoclonal antibody specific for hsp70 (7FB) and stained with an FITC-conjugated second antibody. The cells were then stained with the DNA-binding dye DAPI. (*a,c*). Phase-contrast; (*b,d*) immunofluorescence; (*e*) DAPI staining. No staining was detected in wild-type cells examined under the same conditions.

(Fig. 3b,d). Some cells (e.g., upper right) did not stain at all while others had uniform bright staining. Most showed light diffuse staining and several scattered points of extremely intense staining which, for the sake of simplicity, are hereafter termed hsp70 granules. Often, but not always, these granules surrounded the nucleus. To better localize them, cells were stained with 4′6′-diamidino-2-phenylindole (DAPI). As may be seen in Figure 3, c–e, with short exposures, the protein seems to concentrate just inside the nucleus, in places that do not coincide with regions of high DNA concentration.

The presence of hsp70 granules in *eip300* cells was unexpected and contrasts with previous reports for hsp70 staining in wild-type cells (Velazquez and Lindquist 1984). During heat shock, hsp70 concentrates in nuclei and at cell membranes in *Drosophila* cells. During recovery from heat shock, hsp70 moves to the cytoplasm. With a second heat shock, it rapidly re-enters the nucleus and reconcentrates at cell membranes. Even when concentrated in nuclei and at membranes, the staining in these regions is diffuse, and not granular.

hsp70 staining in wild-type cells during recovery from heat shock

To determine whether hsp70 granules were a peculiarity of *eip300* cells, we re-examined hsp70 localization in wild-type cells. In particular, we examined cells that had been allowed to recover from heat shock for much longer

periods than had been investigated previously (Velazquez and Lindquist 1984). Twelve hours after a 45-min heat shock at 36.5°C, many cells showed conspicuous punctate staining (Fig. 4a), and after 24 hr most did. After 48 hr, many cells no longer stained but, in those that did, staining was primarily restricted to granules (Fig. 4b). The distribution of hsp70 in these cells was much like that in the *eip300* line at normal temperatures, except that at later time points diffuse background staining was reduced. Presumably, this is because most of the *eip300* cells are continuously producing new hsp70, which has not yet coalesced into granules, whereas in wild-type cells hsp70 synthesis is completely repressed during recovery from heat shock.

To ensure that this pattern of localization was not simply an idiosyncrasy of tissue culture cells, late third-instar larvae were subjected to a mild heat shock and allowed to recover at 25°C for 5 hr. Salivary glands were then removed, fixed, sectioned with a cryostat, and reacted with hsp70-specific antibodies. *hsp70* was diffusely distributed in both the nucleus and the cytoplasm (Fig. 4c–e). Punctate staining was superimposed upon the diffuse staining. Again, granules tended to ring the inside of the nucleus. After longer recoveries, larvae pupated and were not examined.

Expression of hsp70 from the metallothionein promoter

The difficulties encountered in obtaining overexpressing transformants and the slow growth of the *eip300* cell line suggest that hsp70 expression is detrimental at normal temperatures. In these cells, as well as in cells that have recovered from heat shock, the coalescence of hsp70 into granules might represent a mechanism for ameliorating the detrimental effects of the protein. To address this possibility in a system more amenable to experimental manipulation, the *hsp70*-coding sequence was placed under the control of the metallothionein promoter, which is inducible by copper. Several stable transformants were readily obtained with this construct (*mt70*). In the absence of copper, these cells grew at the same rate as wild-type cells and did not produce appreciable quantities of hsp70 (Solomon et al. 1991).

To measure the effects of *hsp70* expression on growth, wild-type cells and *mt70* transformants were plated into 96-well microtiter plates at a low density in the absence of drug selection. Cupric sulfate was added to some of the wells at a concentration of 100, 250, or 500 μM. *hsp70* was not induced in wild-type cells but was strongly induced in *mt70* cells (Solomon et al. 1991). After 3 hr of incubation in 500 μM copper, hsp70 accumulated in *mt70* cells to approximately the same level as observed in wild-type cells after a 30-min heat shock at 36°C. In 100 μM copper, the protein accumulated to about one-quarter of this level. The protein produced by these cells was functional in thermotolerance; survival was enhanced when cells were exposed to copper for 3 hr prior to a shift to 41°C (Solomon et al. 1991). Wild-type cells showed no increase in survival under these conditions.

As determined by microscopic examination, wild-type cells doubled within 24 hr of plating at all concentrations of copper used. *mt70* cells doubled within 24 hr when cultured in the absence of copper. In the presence

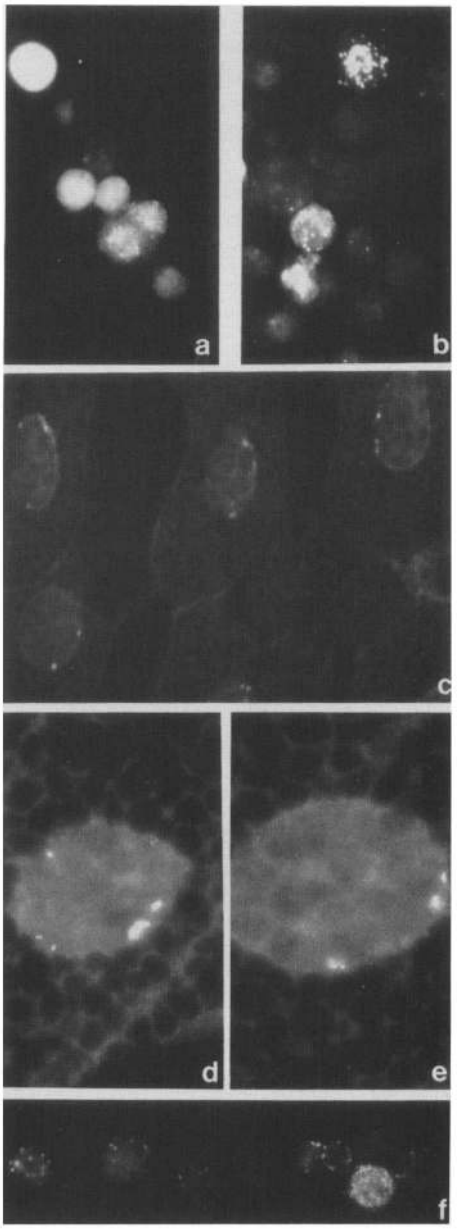

Figure 4. *hsp70* granules in wild-type cells during long-term recovery from heat shock. (*a*,*b*) Tissue culture cells were heat-shocked at 36.5°C for 45 min and returned to 25°C for recovery. After 12 (*a*) or 48 (*b*) hr, cells were fixed and stained as in Fig. 3. (*c*) Larvae were heat shocked at 36.5°C for 45 min and returned to 25°C for recovery. After 5 hr, glands were dissected, sectioned, and stained as in Fig. 3. (*d*,*e*) Higher magnification of salivary gland cells treated as in *c*, showing punctate staining in the periphery of the nucleus. (*f*) *mt70* cells were grown in 500 μM copper for 9 days to induce formation of granules. The cells were treated with actinomycin D to inhibit heat shock protein synthesis and given a stepped heat shock (35°C for 30 min, followed by 39°C for 60 min).

of copper, however, growth rates were reduced. *mt70* cells required 4 days to double in 250 μM copper and required 6 days in 500 μM copper. With continued incubation, growth rates improved. After 7 days of culture, *mt70* cells grew nearly as well as wild-type cells at all copper concentrations (Fig. 5).

The intracellular distribution of hsp70 was determined by indirect immunofluorescence, and accumulation was analyzed by Western blotting. After *mt70* cells had been cultured for 2 days in 500 μM copper, at a time when growth was still inhibited, most cells stained brightly. A small percentage showed little or no staining. Staining was diffuse in all cells that did stain (Fig. 6a). Several days later, when growth resumed, two dramatic changes in hsp70 staining were apparent (Fig. 6c,f): (1) A much greater percentage of cells (~30%) showed no staining; and (2) the distribution of hsp70 had changed in those cells that did stain. Some cells continued to stain diffusely, but most showed bright, distinct granules. In some cases, granules were superimposed on diffuse background staining; in others, staining was restricted to granules. This pattern of staining was very similar to that of the *eip300* cell line. Similar results were obtained in three separate experiments, with three independent *mt70* transformants.

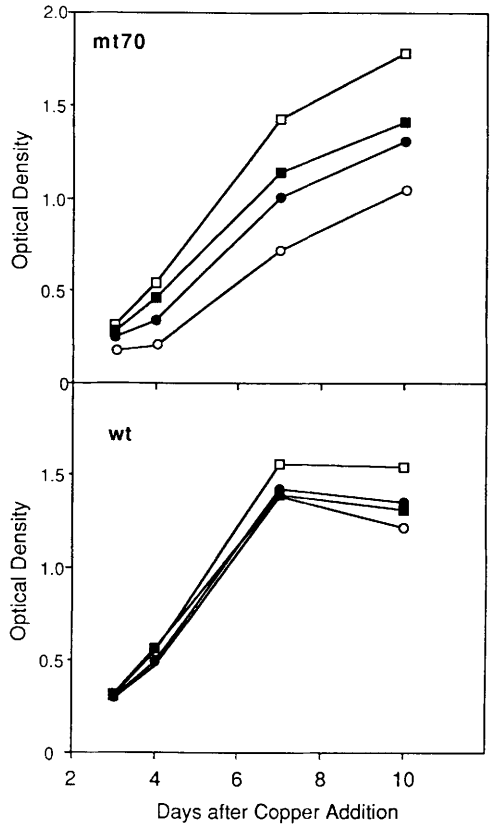

Figure 5. Reduced growth rates in cells expressing hsp70. Wild-type or *mt70*-containing cells were diluted into 96-well microtiter plates with 0 (□), 100 (■), 250 (●), or 500 (○) μM copper. Cell growth was assayed on days 3, 4, 7, and 10 by neutral red staining on the indicated days and quantified by densitometry. Each point represents the average of eight duplicate wells.

Changes in hsp70 staining were accompanied by a reduction in total hsp70 concentrations, as determined by Western blotting. For example, after 9 days of culture in 500 μM copper, hsp70 concentrations were reduced to one-third of earlier levels (data not shown). Together, these results suggest that the resumption of growth is the result of the appearance of two types of cells: cells that have lost the ability to express hsp70, and cells that have sequestered it into granules.

Expression of hsp70 from the salivary gland glue promoter

To examine the effects of constitutive hsp70 expression in a normal *Drosophila* tissue, the *hsp70*-coding sequence was placed under the control of a tissue-specific promoter and integrated into the *Drosophila* genome by P-element-mediated transformation. We chose the promoter of the *salivary glue secretion-3* (*Sgs-3*) gene to express hsp70 in the secretory cells of the salivary gland. The cells of this tissue stop dividing at the end of embryonic development. Thereafter, chromosomes replicate without separation of daughter strands, forming giant polytene cells. The *Sgs-3* promoter is active in the third-larval instar, after the chromosome replications are complete but before the glands have reached maximum size. Thus, this tissue affords an opportunity to monitor the effects of hsp70 on cell growth, separate from cell division.

Two lines carrying independent insertions of the *sgs70* gene were examined. In both lines the glands of homozygotes expressed hsp70 at higher levels and were smaller in size than the glands of heterozygotes or the wildtype. Because gland size can vary with age and culture density, we created salivary glands that were mosaic for hsp70 expression.

To produce mosaics, the *sgs70* gene was placed between target sequences for the site-specific FLP recombinase of *Saccharomyces cerevisiae*, and flies carrying such constructs were crossed to flies carrying a *FLP* recombinase gene under the control of a heat shock promoter (Golic and Lindquist 1989). Embryos were given a mild heat shock to induce mosaicism early in embryogenesis, while the salivary gland primordium was being determined. This treatment had no adverse effects on development but produced glands that were potential mosaics for *hsp70* expression. Actual expression of *hsp70* did not occur until the *Sgs-3* promoter was activated 3 days later.

Three levels of hsp70 expression were observed. This pattern is typical of the mosaicism produced by the FLP recombinase under mild induction conditions (Golic and Lindquist 1989). The nuclei of all cells were of similar size (Fig. 7). This was expected, because the *Sgs-3* promoter is induced only after polytenization is complete. The sizes of whole cells, however, varied dramatically. Cells that did not express hsp70 were large and had the

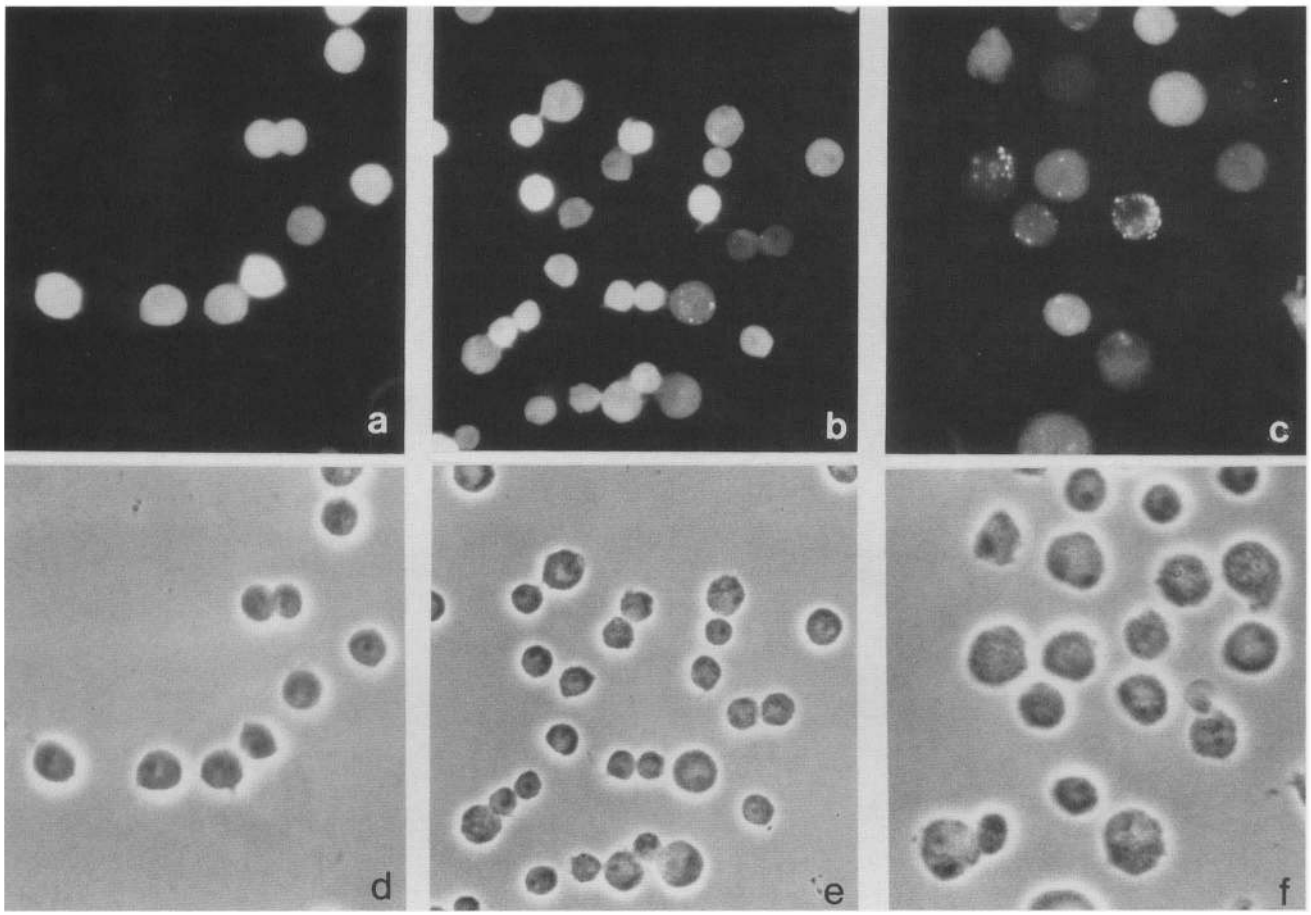

Figure 6. The resumption of growth in copper coincides with the appearance of cells with reduced or granular hsp70 staining. *mt70* cells growing in microtiter dishes in the presence of 500 μM copper, as described for Fig. 5, were harvested on day 2 (*a*,*d*), day 5 (*b*,*e*), or day 9 (*c*,*f*) and stained with monoclonal antibody 7FB, specific for hsp70 and FITC-conjugated secondary antibody. (*a–c*) Phase contrast; (*d–f*) immunofluorescence.

morphology typical of salivary glands at this stage in development. Cells that expressed low levels of the protein showed primarily nuclear and membrane staining and were somewhat reduced in size. Cells that expressed hsp70 at high levels were severely affected. They showed staining both in the nucleus and in the cytoplasm, and their size was much reduced. By adjusting the plane of focus during the examination of these sections, it was clear that all expressing cells also contained hsp70 granules, primarily in the nuclei. The granules are not clearly evident in Figure 7, because the glands also have a high level of diffuse background staining. This is presumably because most of the newly expressed protein from the *Sgs-3* promoter had not yet coalesced into granules.

hsp70 cannot be redeployed from granules

As granules might represent a mechanism for storing hsp70 for later use, we asked whether hsp70 could be remobilized from these granules by heat shock. To produce granules, cells were heated at 36°C for 30 min and allowed to recover for 24 and 48 hr at 25°C. Actinomycin D was then added to block the synthesis of new heat shock proteins, and cells were reheated for 30 min at 37°C. Cells that were reheated were indistinguishable from controls maintained at 25°C with respect to the number, size, and location of granules. Furthermore, an increase in diffuse nuclear or membrane staining was not observed. Similar results were obtained when cycloheximide instead of actinomycin D was employed to block new heat shock protein induction. Sudden severe heat shocks (39°C for 120 min) and stepped heat shocks (35°C for 30 min followed by 39°C for 60 min) also failed to induce the dispersal of granules (Fig. 4f). Finally, when cells containing granules were allowed to synthesize new hsp70 (in the absence of inhibitors), diffuse staining was simply superimposed on the pre-exisiting, granular pattern. These experiments were repeated with *mt70* cells. In this case, granules were produced by incubating cells in copper for 7–9 days. The granules of these cells were as refractory to dispersal as those of cells recovering from heat shock.

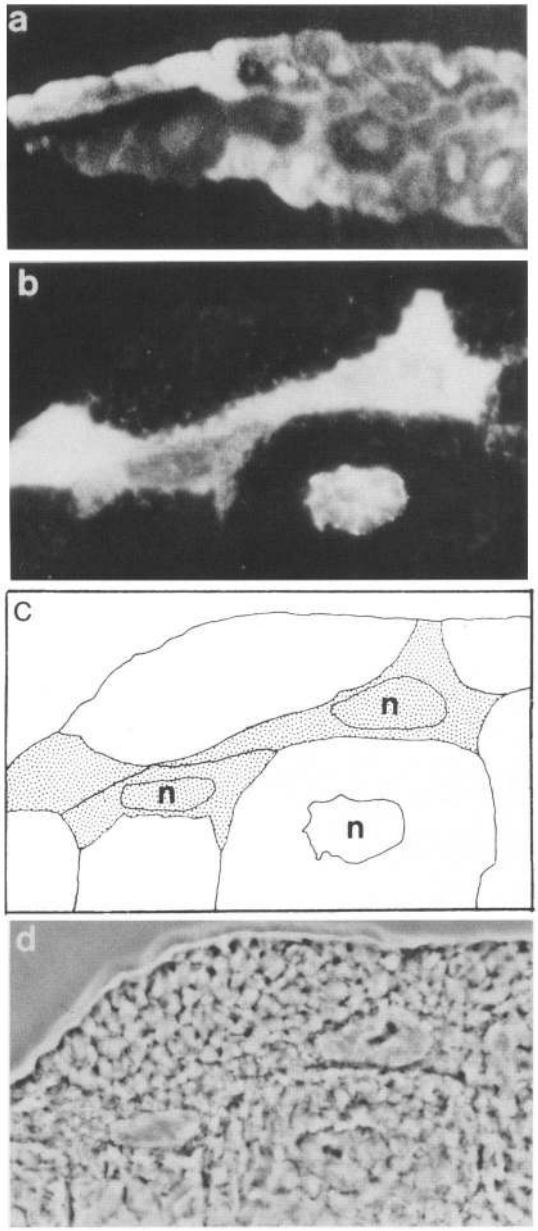

Figure 7. Salivary glands with mosaic expression of hsp70. (*a*) Immunofluorescent detection of hsp70 in a portion of a salivary gland showing three levels of expression: intensely staining cells with two copies of the *hsp70* gene (small cells in *upper left*), moderately staining cells with one copy (cells at *right*), and unstained cells lacking the *hsp70* gene (large cells at *lower left*). hsp70 was visualized by use of the specific monoclonal antibody 7FB and FITC-conjugated secondary antibody. Control larvae, which had been given a similar heat shock early in embryogenesis but did not contain the *sgs70* gene, showed no hsp70 staining at this stage. (*b–d*) Higher magnification of another mosaic gland displaying cells with two levels of hsp70 expression (one or two copies of the gene). (*b*) Immunofluorescence; (*c*) outline drawing, n = nucleus; (*d*) phase-contrast.

We then asked whether the protein in these granules could function in thermotolerance. Cells were heat shocked at 36°C for 45 min and allowed to recover for 30 min, 12 hr, 24 hr, or 48 hr. They were then exposed to 41.5°C for 30 min and growth was monitored over the next 5 days. Cells that had been allowed to recover for only 30 min rapidly resumed growth after the severe heat shock. Granules themselves did not have an adverse effect on thermotolerance. Cells that contained granules in addition to diffusely distributed protein (at 12 hr) were nearly as thermotolerant as cells that had been allowed to recover for only 30 min. However, cells that had been allowed to recover for 24 or 48 hr behaved identically to cells that had not been given any pretreatment, and growth was severely impaired.

By Western blot analysis of one-dimensional gels, the hsp70 protein in cells that had recovered from heat shock for 24 hr had the same electrophoretic mobility as newly synthesized hsp70. Moreover, the concentration of hsp70 protein in these cells was still severalfold higher than that required for full thermotolerance (data not shown; Solomon et al. 1991). We conclude that the protein in hsp70 granules is irreversibly inactivated.

Location of lysosomes and hsp70 granules

A simple explanation for the intense concentration of hsp70 in discrete locations and for the lack of mobilization upon heat shock is that the protein is sequestered in lysosomes, destined for degradation. To investigate the distribution of lysosomes relative to hsp70, tissue culture cells containing granules were incubated with Lucifer Yellow CH, a vital fluorescent dye that is endocytosed and concentrated in lysosomes (Swanson et al. 1987; Holtzman 1989). The cells were then counterstained with hsp70 antibody. Again, we examined granules produced by wild-type cells 24–48 hr after heat shock.

In control cells and in cells containing hsp70 granules, staining with Lucifer Yellow was heterogeneous, with some cells showing few lysosomes and others showing many. As described above, the distribution of hsp70 granules was also heterogeneous. In many cells the two distributions did not overlap at all (Fig. 8, long arrow). In many others, the overlap was only partial (Fig. 8, arrowhead). A close correspondence between hsp70 and Lucifer Yellow staining occurred in only a few cells (Fig. 8, short arrow). Similar results were obtained with *mt70* cells after 7–9 days of incubation with copper. *hsp70* granules may eventually merge with lysosomes, but they can and do arise independently of them.

Discussion

We find that the expression of hsp70 at normal temperatures has a deleterious effect on *Drosophila* cells. Although it does not reduce viability, hsp70 inhibits growth in both tissue culture cells and in the polytene cells of salivary glands. The detrimental effects of the protein on growth contrast with its beneficial effects on survival at high temperatures (Li and Laszlo 1985; Johnston and Kucey 1988; Riabowol et al. 1988; Li et al.

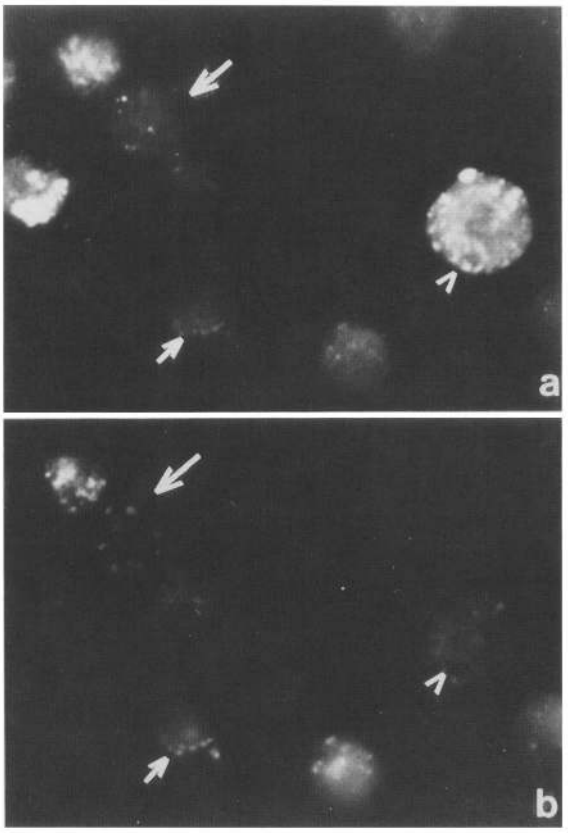

Figure 8. *hsp70* expression and lysosomes in wild-type tissue culture cells during recovery from heat shock. (*a*) Immunofluorescent detection of hsp70 by use of antibody 7FB and goat anti-rat $F(ab')_2$ conjugated to Texas Red. (*b*) Lucifer Yellow staining of lysosomes. Some show no overlap (long arrow) while only a few cells show complete overlap (short arrow). Most cells exhibit only a partial overlap between hsp70 granules and lysosomes (arrowhead).

1991; Solomon et al. 1991). The need to balance these opposing effects explains why so many regulatory mechanisms are employed to control hsp70 expression in *Drosophila*. We suggest that the formation of hsp70 granules represents yet another mechanism for controlling hsp70, in this case repressing its activity rather than its expression.

hsp70 proteins have different expression patterns in different organisms. In *Drosophila*, the major heat-inducible protein hsp70 is virtually undetectable at normal temperatures (Velasquez et al. 1983). In contrast, in both yeast and mammalian cells, heat-inducible hsp70 proteins are expressed at a substantial level at normal temperatures (Milarski and Morimoto 1986; Werner-Washburne et al. 1987). Yet even in yeast and mammalian cells, increasing the basal expression of hsp70 may reduce the rate of growth (Stone and Craig 1990; Li et al. 1991). It is possible that the constitutive and heat-inducible proteins of the nuclear/cytoplasmic compartment are functionally equivalent and it is only the total level of hsp70-related protein that must be controlled. However, in yeast cells, increased expression of certain members of the family is insufficient to rescue the mutant phenotypes of other members (Craig and Jacobsen 1984). Thus, even among the members of the nuclear/cytoplasmic compartment there seems to be considerable specialization in function. Our results provide further evidence of such specialization. The heat-inducible hsp70 of *Drosophila* increases survival during exposure to high temperatures (Solomon et al. 1991) but is actually deleterious at normal temperatures. The basal level of hsp70 expression in our extra copy line at 25°C was only a fraction of the total hsp70-related protein in the cell (Solomon et al. 1991). Nevertheless, the extra copy transformant was very difficult to obtain and, once obtained, was unstable. Similarly, the level of hsp70 expressed from the mt70 promoter was less than the normal constitutive level of hsp70 homologs, yet it reduced growth sharply and dramatically.

Recent work elucidating the biochemical functions of hsp70 proteins provides a framework for understanding the deleterious effects of hsp70 at normal temperatures. Proteins in the hsp70 family participate in a wide variety of protein-folding pathways in the cell (Lindquist and Craig 1988; Gething and Sambrook 1992). For example, they keep precursor proteins in an unfolded, translocation-competent state for transport across membranes. They may also bind newly synthesized, incompletely folded proteins as they emerge from ribosomes, facilitating their assumption of normal structure. In a similar fashion, the heat-inducible proteins are believed to interact with a wide variety of cellular proteins as they become unfolded by elevated temperatures. By preventing unfolded and partially unfolded proteins from aggregating, they facilitate the recovery of normal protein structure and function when cells are returned to normal temperatures (Pelham 1986; Rothman 1989; Skowyra et al. 1990). Thus, hsp70 might dissociate from its substrates slowly, to optimize protective function at high temperatures. In the absence of stress, this same property might reduce rates of growth if the protein binds to the same substrates as constitutive members of the family. Alternatively, the protective role of hsp70 might require binding to a different set of substrates. For example, because heat shock is invariably accompanied by a pause in growth and development in *Drosophila*, hsp70 might interact specifically with growth and cell cycle regulators to produce this pause.

By forcing expression of hsp70 at normal temperatures from an inducible promoter, we were able to examine the consequences of its expression in considerable detail. In no case did we observe a loss in viability, only a sharp reduction in growth. In *mt70* cells, growth inhibition was temporary. When growth resumed, two changes in hsp70 expression were observed: (1) The number of cells that did not express the protein increased; and (2) in those cells that did express the protein, its intracellular distribution changed from diffuse to granular.

We have not investigated the mechanism by which an increase in non-expressing cells occurs. The metallothionein-regulated *hsp70* gene might simply be lost. How-

ever, in cotransfections of the type used here, plasmids carrying the selectable marker and plasmids carrying the *mt70* gene cointegrate into the genome in mixed arrays (Bourouis and Jarry 1983) and are stable for months, even in the absence of selection (J. Solomon and S. Lindquist, unpubl.). Another possibility is a metastable change in chromatin structure or matrix attachment regions that inactivates the transfected gene (Gottschling et al. 1990). Whatever the mechanism, the rapid increase in nonexpressing cells suggests that a strong selective pressure favors their appearance.

We have begun to investigate the process by which the intracellular distribution of hsp70 changes from diffuse to granular. The distribution of hsp70 expressed from the constitutive *eip28* promoter (in *eip300* cells) is similar to the distribution of hsp70 expressed from the *metallothionein* promoter after 9 days of culture in copper (in *mt70* cells). Granules are found throughout the cell, commonly just inside the nucleus. Similar localizations were observed in salivary glands that were expressing the protein from the *Sgs-3* promoter and in wild-type glands during the first 5 hr of recovery from heat shock. In wild-type tissue culture cells, after 24–48 hr of recovery, more of the granules are cytoplasmic; and in some cases their positions coincide with lysosomes. This suggests that there may be a pathway through which the granules move, from nucleus to cytoplasm, eventually to be engulfed by lysosomes for degradation. Alternatively, cytoplasmic granules may form independently of nuclear ones, and lysosomal concentration may proceed independently of granule formation. It is clear, however, that the intense concentration of hsp70 into discrete points in the cell is not simply the result of lysosomal targeting.

A particularly intriguing possibility is that coalescence is an inherent property of the protein itself. At high temperatures, hsp70 presumably interacts with a large number of proteins as they become denatured. When high-affinity binding sites exposed by stress are not available, 70 molecules may interact with other hsp70 molecules to form larger complexes. Under certain conditions in vitro, mammalian hsp73 forms oligomers of 300 kD or greater (Palleros et al. 1991). These oligomers do not bind ATP and are resistant to dissociation even when incubated with additional hsp73 and ATP. Thus, although hsp70 proteins can catalyze the disaggregation of other proteins, hsp73 oligomers are not a substrate for this reaction. The properties of these in vitro oligomers are reminiscent of the hsp70 granules described here. The accumulation of protein in granules is irreversible, even when cells are given a second heat shock and additional hsp70 protein is expressed. Thus, although hsp70 may play a role in disaggregating certain proteins (Skowyra et al. 1990), hsp70 granules are not a substrate.

Clearly, granules do not provide a means for storing hsp70 for use in a subsequent heat stress. Perhaps the deleterious effects of the protein on normal growth warrant inactivation rather than storage. But why isn't the protein simply degraded? One possibility, suggested by recent experiments with embryos, is that sequestration is a regulated process and can proceed with remarkable speed under certain biological circumstances. When *Drosophila* embryos are given a short pretreatment at 36°C, they acquire tolerance to a subsequent severe heat shock. In older embryos recovering from heat shock at 25°C, tolerance is lost gradually and this loss roughly parallels the loss of hsp70. Similar phenomena have been observed in many organisms and cell types (Li and Laszlo 1985; Nover 1991). In marked contrast, early postblastoderm embryos lose induced tolerance with extraordinary speed. Within 10 min of return to 25°C, at a time when hsp70 concentrations are still high, thermotolerance is virtually extinguished (M. Welte, J. Feder, and S. Lindquist, unpubl.). In these early embryos, but not in later embryos, hsp70 is largely sequestered into granules within 10 min of return to 25°C . Another major difference between these two stages is that early embryos, but not late embryos, are characterized by rapid rates of cell division. The rapid sequestration of hsp70 may be required to re-establish normal patterns of cell growth and division during recovery. It is as though the early embryo would rather risk lethality from a second heat shock than maintain an active pool of hsp70. At yet earlier stages, an even more extreme position is taken. In preblastoderm embryos, where nuclei divide in a syncytium at the remarkable rate of once every 10 min, hsp70 cannot be induced by heat shock. Moreover, while other heat shock proteins are synthesized by ovarian nurse cells and transported into the egg, hsp70 is not (Zimmerman et al. 1983). The embryo is extremely thermosensitive at this stage and why it denies itself the protective effects of hsp70 has long been a puzzle. The inhibitory effect of hsp70 on growth at 25°C provides a simple explanation. We are currently testing these hypotheses with promoters that should provide hsp70 expression in eggs and early embryos.

Materials and methods

Plasmid construction

p400, *p110*, and *pmths70* (Solomon et al. 1991) were transformed into Schneider's line 2 (SL2) to create the lines *hsp70-P*, *hsp70Δ-P*, and *mt70*, respectively. *p400* contains the regulatory and coding sequences of hsp70 with a 50-bp oligonucleotide encoding 14 amino acids inserted after the last codon of *hsp70*. This carboxy-terminal extension of the protein encodes an 8-amino-acid hydrophilic spacer, followed by 6 substance P amino acids that are recognized by antibody NCI/34 (Cuello et al. 1979). *p110* is the same as *p400* except that sequences between *Ava*I and *Bam*HI are removed, resulting in an in-frame deletion producing a smaller protein. *pmths70* contains 430 bp of the *metallothionein* promoter (Maroni et al. 1986) driving the complete *hsp70*-coding sequence.

eip300 contains the entire *hsp70*-coding sequence and 3′ sequences to +2401 fused to the promoter derived from the ecdysone-inducible genes *Eip28* and *Eip29*. This derivative is constitutive and not responsive to ecdysone. *eip300* was constructed from plasmids *pDM300* (wild-type *hsp70*-coding sequences; McGarry and Lindquist 1985) and *pC44*, provided by R. Petersen. To construct *pC44*, the *Bam*HI–*Eco*RI fragment of *pDM110* (McGarry and Lindquist 1985) was ligated to the

*Bam*HI–*Eco*RI fragment of *pEIPPr650* (Cherbas et al. 1986). The resulting plasmid contains 650 bp of the *EIP28/29* promoter region and transcribed sequences to +11 joined to the *hsp70* gene. This intermediate carried a partial deletion of the *hsp70*-coding sequence, which was restored by ligating the *Cla*I–*Eco*RI fragment of *pC44* to the *Cla*I–*Eco*RI fragment of *pDM300*, creating *eip300*.

sgs70 was derived from *pDM420* (McGarry and Lindquist 1985; McGarry 1986), which carries a *Bam*HI linker insertion in the *hsp70* leader at +231 (−10 with respect to the start site of translation) and a deletion in the protein-coding region. *pDM420* was cut with *Hin*dIII and *Bam*HI, and the fragment containing the *hsp70*-coding sequence was gel purified, filled in with Klenow, and ligated to *Kpn*I linkers (New England Biolabs). This intermediate was digested with *Cla*I and *Eco*RI to remove the *hsp70*-coding deletion, and the *Cla*I–*Eco*RI fragment from *pDM300* was inserted (as in the *eip300* plasmid). This plasmid, *pK420*, was cut with *Kpn*I and treated with calf intestinal phosphatase. A 2.4-kb *Kpn*I fragment from *pGOA2.98* (Roark et al. 1990), including the *Sgs-3* promoter, TATA box, mRNA start site, and 12 bp of the *Sgs-3* leader sequence, was ligated to the *Kpn*I site of *pK420*. The *Bgl*II–*Eco*RI fragment containing the *Sgs-3* 5′ sequences fused to *hsp70*-coding and 3′ sequences was ligated into the shuttle vector *pHSS6* (Siefert et al. 1986), which had been cut with *Bgl*II and *Eco*RI to provide *Not*I linkers on either side of the fusion gene. A *Not*I fragment from this construct was ligated into $pP[>w^{hs}\cdot N>]$, which contains a *Not*I restriction site inserted in place of the *Hin*dIII site of $pP[>w^{hs}>]$ (Golic and Lindquist 1989).

Cell culture and transformation

SL2 cells were grown in modified Shields and Sang media with 10% fetal calf serum (S&S + FCS) (DiDomenico et al. 1982). For extended heat shock recovery experiments, wild-type cells were heat shocked by immersing the culture flask in a circulating water bath maintained at 36.5°C. Flasks were returned to 25°C for recovery. Thermotolerance assays were performed as described previously (Solomon et al. 1991).

Transformed cell lines were obtained by the method of Wigler et al. (1979), as modified by Rubin (Ashburner 1989). Transformed cell lines were maintained in S&S + FCS supplemented with 200 μg/ml of hygromycin B (for selection with bacterial hygromycin B phosphotransferase) or 0.2 μM methotrexate (for selection with bacterial dihydrofolate reductase).

For induction of hsp70 in *mt70*, tissue culture cells were diluted to 2.5×10^5 cells/ml in fresh medium, and cupric sulfate was either omitted or added to a final concentration of 100, 250, or 500 μM. At 500 μM, expression of hsp70 in *mt70* cells is near maximal; at 100 μM, expression is roughly one-quarter of the 500 μM level. Copper does not induce heat shock proteins in wild-type cells at concentrations below 1000 μM under these culture conditions, possibly because proteins in the serum chelate much of the copper. Fifty-microliter portions of each culture were distributed to 96-well tissue culture plates. Every plate contained eight duplicate wells for each copper concentration and each cell type. Sufficient plates were seeded to provide one full plate for each assay each day. The cells were grown at 25°C in humid chambers. On various days after copper addition, cell growth was assayed by microscopic examination and by neutral red staining. Neutral red assays are described in Solomon et al. (1991).

Electrophoretic analysis of proteins

Proteins were pulse labeled with [^{3}H]leucine, as described by DiDomenico et al. (1982). Total proteins were collected from equal quantities of cells by the addition of ice-cold 10% TCA, followed by centrifugation at 2800G. Proteins were rinsed twice with ethanol, desiccated, and resuspended in sample buffer (for direct application to a gel) or in 100 μl of 1% SDS for analysis by the Pierce BCA protein assay with the enhanced protocol described by the manufacturer.

For Western blots, electrophoretically separated proteins (DiDomenico et al. 1982) were transferred to nitrocellulose or Immobilon membranes (Towbin et al. 1979). Immobilon blots were stained with Coomassie blue (to demonstrate equal protein loading). All blots were blocked with 5% nonfat dry milk and calf serum. Peptide-tagged proteins were amidated (Sheehan and Hess 1955) for recognition by monoclonal antibody NC1/34 (Cuello et al. 1979), reacted with rabbit anti-rat IgG, then with horseradish peroxidase-conjugated goat anti-rabbit IgG, and visualized enzymatically with 4-chloro-1-napthol. hsp70 from eip300 and mt70 was reacted with monoclonal antibody 7FB, specific for hsp70 (Velazquez et al. 1980). Protein from eip300 was visualized as for the tagged protein. Protein from mt70 was identified by use of rabbit anti-rat IgG, followed by goat anti-rabbit IgG and, finally, 3 μCi of ^{125}I-labeled protein A. Autoradiographic exposures, quantified with an LKB laser densitometer, were within the linear range of film response, as determined by scanning films containing a dilution series of heat shock protein.

Cytological analysis of Drosophila *cells and tissues*

For immunological localization of hsp70, tissue culture cells were collected by gentle pipetting, washed with 1 ml of S&S minus serum, and collected by centrifugation in an IEC model CL clinical centrifuge at setting 3 for 1 min. Cells were resuspended in 100 μl of S&S without serum and transferred to eight-chambered glass microscope slides. Adhesion was encouraged by very gentle centrifugation, and the slides were incubated at room temperature for 15 min. In some experiments cells were grown in chamber slides overnight.

For experiments with salivary glands, the tissue was dissected from wandering third-instar larvae in PBS, transferred to a drop of O.C.T., frozen to −57°C on the quick freeze bar of a Slee Cryostat, allowed to equilibrate to the sectioning temperature of −20°C and 4 μM sections were cut (Hafen and Levine 1986). Both tissue culture cells and salivary gland sections were fixed with 4% paraformaldehyde in PBS for 15 min. Cells and sections were rinsed three times in PBS, treated with 100 μl of TS–PBS (0.5% Triton X-100 and 10% calf serum in PBS), incubated with 7FB (ascites diluted 1/500 in TS–PBS), rinsed three times in PBS, rinsed once with TS–PBS incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat $F(ab')_2$ (Cappel) diluted 1/300 in TS–PBS, washed with TS–PBS, rinsed three times with H_2O, and dried. To identify nuclei, cells were stained with DAPI (1 μg/ml), after reaction with antibodies. All preparations were mounted in freshly prepared 70% glycerol in PBS containing 1 mg/ml of *p*-phenylenediamine (Johnson and de C. Nogueira Araujo 1981). Phase-contrast and epifluorescent images were recorded with Technical Pan 2415 (ASA 100) or Tri-X pan (ASA 400) film.

To determine whether preformed hsp70 granules redisperse during heat shock, *mt70* cells were preinduced with copper for 9 days or wild-type cells were heat shocked at 36.5°C for 45 min and allowed to recover at 25°C for 24 or 48 hr. Cells were then treated with actinomycin D (10 μg/ml) or cycloheximide (20 μg/ml) to block further heat shock protein induction and shifted to 35°C for 30 min, to 39°C for 2 hr, or to 35°C for 30 min, followed by 39°C for 90 min.

To localize lysosomes, cells were incubated with Lucifer Yel-

low CH (final concentration, 0.5 mg/ml) in tissue culture flasks for 8 hr, rinsed and resuspended in S&S + FCS, and incubated for 2 hr at 25°C. Cells were then stained for hsp70 as described above except that the secondary antibody was Texas Red-conjugated goat anti-rat $F(ab')_2$ (Jackson Immunoresearch). Cells were examined with both 435 nm excitation (for Lucifer Yellow CH) and 546 nm excitation (for Texas Red).

Fly culture, transformations, and heat shock

Flies were maintained on standard cornmeal–agar media in a 25°C incubator. The *sgs70* construct was transformed into the germ line of *D. melanogaster* by standard techniques of P-element-mediated transformation (Rubin and Spradling 1982; Spradling and Rubin 1982), by use of helper plasmid p25.7πwc (Karess and Rubin 1984) and w^{1118} embryos. Surviving G_0 individuals were individually mated to w^{1118} virgins, and transformants were selected as offspring with orange eyes. Homozygous lines were made by inbreeding. Mosaics were produced by mating homozygous flies to w^{1118} hsFLP1 flies (carrying the FLP recombinase). Vials containing 5- to 7-hr old heterozygous embryos were immersed in a 37°C temperature-controlled circulating water bath for 1 hr to induce FLP-mediated recombination (Golic and Lindquist 1989).

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The consequences of expressing hsp70 in Drosophila cells at normal temperatures.

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