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EXPERIMENTAL EVOLUTION OF HSP70 EXPRESSION AND THERMOTOLERANCE IN *DROSOPHILA MELANOGASTER*

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Abstract.—To examine whether recent evolutionary history affects the expression of Hsp70, the major heat-induced-heat shock protein in *Drosophila melanogaster*, we measured Hsp70 expression, thermotolerance, and *hsp70* gene number in replicate populations undergoing laboratory evolution at different temperatures. Despite Hsp70's ancient and highly conserved nature, experimental evolution effectively and replicably modified its expression and phenotype (thermotolerance). Among five *D. melanogaster* populations founded from a common ancestral population and raised at three different temperatures (one at 18°C, two each at 25°C and 28°C) for twenty years, Hsp70 expression varies in a consistent pattern: the replicate 28°C lines expressed 30–50% less Hsp70 than the other lines at a range of inducing temperatures. This modification was refractory to acclimation, and correlated with thermotolerance: the 28°C lines had significantly lower inducible tolerance of 38.5°C and 39°C. We verified the presence of five *hsp70* genes in the genome of each line, excluding copy number variation as a candidate molecular basis of the evolved difference in expression. These findings support the ability of Hsp70 levels in *D. melanogaster* populations to change over microevolutionary time scales and implicate constancy of environmental temperature as a potentially important selective agent.

Key words.—Acclimation, *Drosophila*, experimental evolution, heat-shock proteins, Hsp70, thermotolerance.

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A principal contribution of the experimental evolution approach (Rose et al. 1996) is its ability to dissect the mechanisms of evolution in action. Whereas the dynamics, repeatability, and sources of evolutionary variation can be difficult to discern in natural populations, experimental evolution can examine these features directly. This approach has been particularly successful in the analysis of physiological evolution, including temperature adaptation of *E. coli* via temperature-specific differences in glucose transport (Bennett and Lenski 1996), the role of larval lipid uptake in *Drosophila* starvation resistance (Chippindale et al. 1996), and metabolic factors underlying the trade-off between *Drosophila* early fecundity and longevity (Djawdan et al. 1996). Extending this technique to examine the expression of genes relevant to physiological adaptation may be especially useful in determining how mechanistic/molecular changes result in physiological trait evolution. Here we examine the experimental evolution of Hsp70 expression and thermotolerance and a candidate molecular explanation for this evolution, change in *hsp70* copy number.

Hsp70, the major heat-inducible heat-shock protein in *Drosophila melanogaster*, is an extremely ancient and conserved protein/gene. All five kingdoms of life possess genes encoding Hsp70 family members (Gupta and Singh 1994), with extremely high sequence similarity among very distantly related organisms (e.g., 74% amino acid sequence identity between mouse and *Drosophila* Hsp70). Hsp70's function in cellular/organismal thermotolerance is also apparently conserved in many species: Hsp70 expression promotes thermotolerance in *Drosophila* (Welte et al. 1993; Feder et al. 1996), fish (dIorio et al. 1996), mammalian cell lines (Li et al. 1995), plants (Lee and Schoffl 1996), and fungi (Hottiger et al. 1992). Although Hsp70's relative contribution to the heat-shock response of different organisms can vary, key regulatory features are conserved across species (e.g., promoter sequence and organization [Fernandes et al. 1994], transcription factor utilization [Wu 1995], and autoregulatory feedback [Lindquist 1993]); a fundamental pattern of stress-inducible, temporally limited expression is basically universal. Thus, Hsp70 is an ancient and apparently immutable component of a critical physiological trait, which suggests that it might be fixed in natural populations and refractory to microevolutionary modification. Yet, despite the highly symplesiomorphic nature of Hsp70 and its phenotype, it clearly varies in nature, even within single populations. For example, Hsp70 expression varies substantially within natural *Drosophila* populations and correlates with inducible tolerance of severe heat shock (Krebs and Feder 1997a). Obviously many evolutionary mechanisms, including rapid adaptation to local conditions, could generate such variation. Accordingly, we asked whether experimental evolution at different temperatures in the laboratory would yield a similar outcome.

We exploited a unique long-term experimental evolution experiment: five *D. melanogaster* populations founded from a single Oregon R line more than 20 years ago and maintained at different but nonextreme temperatures. Pairs of replicate lines have been maintained at 25°C and 28°C, and one line at the ancestral 18°C. Laboratory natural selection has affected numerous traits in these lines, including body size and shape (Cavicchi et al. 1985, 1991), the thermal sensitivity of fitness traits (Cavicchi et al. 1989), and importantly, adult thermotolerance (Cavicchi et al. 1995).

The focus of the present study is on Hsp70 expression curves, that is, the functions relating Hsp70 expression to temperature of heat shock. In these curves, expression is maximal at some intermediate temperature and low to nonexistent at both low and very high temperatures. These curves

bear on several issues. Do expression curves differ among the experimental lines in their position and/or magnitude or are they fixed in the face of experimental evolution? We might expect experimental evolution at high temperature to increase Hsp70 expression as it appears to have done in nature, where Hsp expression often varies according to thermal niche (Feder and Hofmann 1999). However, the costs of increased Hsp70 expression (Krebs and Feder 1997b, 1998) may limit such evolution. Next, to what extent are differences among the lines phenotypic responses to rearing temperature or fixed evolutionary outcomes? To distinguish these alternatives, we characterized each line's expression curve both when taken from its native rearing environment and after acclimation to a common temperature for one generation. Finally, the previous evidence linking Hsp70 and thermotolerance suggests that expression pattern should be correlated with inducible thermotolerance. Therefore, we analyzed and compared each line's inducible thermotolerance with its Hsp70 expression curve.

We also investigated whether variation in *hsp70* gene copy number might underlie variation in Hsp70 expression. The haploid *D. melanogaster* genome typically includes five *hsp70* genes. However, polymorphism for *hsp70* copy number apparently exists within common *Drosophila* laboratory strains (Ish-Horowicz et al. 1979a). Work with both chromosomal deletions (Ish-Horowicz et al. 1979b; Udvardy et al. 1982) and transgenically introduced extra *hsp70* copies (Welte et al. 1993) establishes that *experimental* alteration of copy number can decrease and increase transcription level, respectively. Because *hsp70* mRNA level in heat-shocked adults varies among the experimental lines (Cavicchi, unpubl. data), we asked: Did *evolutionary* alteration of copy number affect *hsp70* transcription and thus expression?

Materials and Methods

Origin of Populations

The experimental populations were founded in 1978 from an Oregon R stock that had itself been maintained at 18°C for at least 20 years (Cavicchi et al. 1995; details in Cavicchi et al. 1985, 1989). Briefly, eggs from 55 individual pair matings were collected and used to found three mass populations, each kept at a different temperature: 18°C, 25°C, and 28°C. Each population was subsequently replicated using eggs from F_1 females. One 18°C line was lost five years after founding. Throughout the history of the experimental lines, multiple random pairs have been used to found each generation and population size has been kept large to minimize inbreeding and drift. Since October 1996, we have maintained each line in a minimum of four standard bottles, keeping population size high (> 1000). The one 18°C line has evolved a sex ratio of roughly 5:1 males:females, which necessitates occasional culling of males. Fecundity and egg-to-larval survival are lower in this line than the others; sample sizes in thermotolerance experiments are uneven due to the resulting low yield of larvae for analysis. The replicate 25°C and 28°C lines are referred to as "A" and "B," that is, A25, B28.

Measuring Hsp70 Expression

We measured Hsp70 expression in larvae, the developmental stage most likely to experience heat shock in nature (Feder et al. 1997). Third-instar larvae were placed in pairs in 1.5-ml cryotubes with 10 μl PBS to humidify, and the tubes were submersed in circulating water baths thermostatted to the heat-shock temperature for one hour. After one-hour recovery at 25°C, tubes were placed in liquid nitrogen and then stored at −80°C. Whenever possible, samples were blocked evenly across heat shock temperature, line, and date.

Hsp70 expression level was measured as described previously (Welte et al. 1993; Feder et al. 1996). Hsp70 concentration was estimated with an enzyme-linked immunosorbent assay (ELISA). Concentrations are expressed as percentages of an Hsp70 standard prepared from *Drosophila* cells in culture.

Basal and Inducible Thermotolerance

All lines were acclimated to 25°C for one generation, and newly eclosed adults were allowed to oviposit on petri dishes containing yeasted standard medium. Each day's plate yielded developmentally synchronized larvae. Third-instar larvae were extracted in about 3 M NaCl. Larvae were transferred in groups of 20 into standard glass rearing vials containing about 7 mL media. Vials were plugged with rubber stoppers and submerged in thermostatted circulating water baths according to treatment. After heat treatment, stoppers were removed and replaced with cotton, and vials were placed at 25°C. Survival was scored as successful adult eclosion and is reported as a percent normalized to mean control survival (larvae in vials that were prepared as above but kept at 25°C for 3 h).

A preliminary experiment characterized the basal thermotolerance of the A25 line. Groups of four vials were prepared as above and treated for one hour at 36°C, 37°C, 38°C, and 39°C. From these results and the analysis of Hsp70 expression, we chose 36°C as a pretreatment temperature that would induce near-maximal levels of Hsp70 without causing mortality. We then analyzed tolerance of one hour at 38.5°C or 39°C in all lines, either with or without pretreatment (1 h at 36°C and 1 h at 25°C). Sample sizes were matched in treatment groups. No significant effects were found due to date or water bath.

Statistics

Statistical analyses used StatView 4.5 (Abacus Concepts Inc., Berkeley, CA). The lack of a replicate 18°C line precluded a nested analysis. All analyses of variance in Hsp70 expression tested simply for line effect (rather than line nested within selection temperature). For Hsp70 expression curve analysis, the design was balanced ($n = 4$ samples for each line/temperature). Factorial ANOVA was used to calculate line, temperature, and line × temperature interactions. Sample sizes for thermotolerance were unbalanced due to the problems with the single 18°C line (see above); statistical analysis was restricted to simple ANOVA (line effect alone).

Determining hsp70 Copy Number

The number of *hsp70* genes in the genome of each line was determined via Southern blotting. This technique has determined *hsp70* copy number in several *Drosophila* species

TABLE 1. Analyses of variance of effect on Hsp70 expression of heat-shock temperature (temp), acclimation to 25°C (acc), and replicate line (line, in 28°C ANOVA only).

Source	df	SS	MS	*F*-ratio	Prob > *F*
18°C line					
Temp	5	30,093.087	6018.617	30.5730	<0.0001
Acc	1	638.576	638.576	3.2438	0.0771
Temp × acc	5	3066.115	613.223	3.1150	0.0150
Residuals	56	11,024.185	196.860		
28°C line					
Temp	5	22,432	4486.4	82.3847	<0.0001
Acc	1	60.9313	60.9313	1.1189	0.2927
Line	1	4.5844	4.5844	0.0842	0.7723
Temp × acc	5	349.91	69.9819	1.2851	0.2764
Acc × line	1	126.059	126.059	2.3148	0.1313
Temp × acc × line	5	132.331	26.4663	0.4860	0.7860
Residuals	5	106.296	21.2592	0.3904	0.8544
	99	5391.215	54.46		

(Leigh Brown and Ish-Horowicz 1981). Digestion of genomic DNA by the restriction enzymes BamH1 and Bgl1 yield diagnostic fragments of 3.15 kb, 2.8 kb, 1.6 kb, 1.5 kb, and 1.45 kb, each possessing one *hsp70* gene (Holmgren et al. 1979).

An *hsp70*-specific labeled probe was generated from the plasmid pdM300 (McGarry and Lindquist 1986), which contains one *D. melanogaster hsp70* gene. DIG-PCR amplification using the Genius kit (Boehringer-Mannheim) yielded a digoxygenin-labeled probe of about 1 kb, comprising the 3′ half of the *hsp70* coding sequence (Primers, upper: 5′-AAAGTAAGCCGTGCCAGGTT-3′; lower, 5′-CCAGAGTAGCCGCCAAATCC-3′). Genomic DNA was extracted from groups of 70 young adults of each line as needed using a standard phenol/chloroform extraction protocol, resuspended in 50 μl Tris-EDTA buffer, and stored at −20°C. DNA concentration was estimated spectrophotometrically. Restriction digestion of genomic DNA with BamH1 and Bgl1 (Promega) was conducted according to the supplier's instructions for 4 h at 37°C. Approximately 10 μg of genomic DNA was digested, reprecipitated with Qiagen P3 solution and absolute ethanol, resuspended in 10 μl TE buffer, and loaded on 1.5% agarose gels. Following electrophoresis for approximately 3 h at 100 V, DNA was transferred onto Nytran nylon membranes using the TurboBlotter system (Schliecher and Schuell) according to the manufacturer's protocol for neutral transfer of DNA. Southern blotting was conducted with the Genius system (Boehringer-Mannheim) following the manufacturer's protocol, using the DIG-PCR product as probe. Hybridization was detected via the NBT/X-phosphate colorimetric method, and allowed to develop for 2–3 h. Following quenching with distilled water, blots were scanned on a UMAX flatbed scanner for computer storage and printing.

PCR Screening

Gene-specific PCR verified *hsp70* copy number in individual flies. Primers to specifically amplify the promoter regions of each *hsp70* gene were designed using the OLIGO software package and published DNA sequence data available from GenBank (accession numbers K01292, K01293, J01104, J01105). PCR amplification using primers Hsp70Bb-U (5′-ATTGGTTTAGAACGCCAGTA-3′) and Hsp70Bb-L (5′-TTGATGGATAGGTTGAGGTT-3′) yields a single product of 1.7 kb, comprising the 5′ flank, promoter area, and partial coding sequence of *hsp70Bb*. Hsp70Bc-U (5′-ATTTGGCGGCTACTCTGGAC-3′) and Hsp70Bc-L (5′-ACTGTGTTTCTGGGGTTCAT-3′) yield a single product of 1.6 Kb, containing the *hsp70Bb-Bc* intergenic region, *hsp70Bc* promoter, and partial coding sequence. For our analysis, DNA was extracted from individual flies according to Gloor et al. (1993). PCR was conducted using Fisher brand *Taq* Polymerase in 25-μl volumes; 5 μl of each reaction was loaded on 2% agarose gels stained with ethidium bromide and visualized under ultraviolet light.

RESULTS

Acclimation

Acclimation to 25°C for one generation affected neither the magnitude or position of Hsp70 expression curves in the 18°C and 28°C lines (Table 1, Fig. 1). For the 18°C line, ANOVA reveals no overall effect of acclimation, but a significant acclimation × temperature interaction ($P = 0.015$, see Table 1). This effect was marked at 35°C and 38°C, whereas the 18°C line responded to acclimation in an inconsistent fashion: acclimation decreased expression at 35°C and increased expression at 38°C (post hoc Bonferroni-Dunn tests find a significant [$P < 0.005$] effect of acclimation at both temperatures).

Comparison among All Lines

Hsp70 expression varied significantly among the experimental lines, with a significant line × test temperature interaction (Table 2); the effect of experimental evolution varied at different points along the Hsp70 expression curves. Indeed, selection temperature altered Hsp70 expression curves in a characteristic and repeatable fashion: at 34–37°C, the 28°C lines expressed 30–50% less Hsp70 than any other line (Fig. 2). At 33°C, 34°C, and 35°C, the 18°C line expressed more Hsp70 than the other lines; however, this difference was sensitive to acclimation (Fig. 1). Expression curves of replicate lines are statistically indistinguishable (post hoc

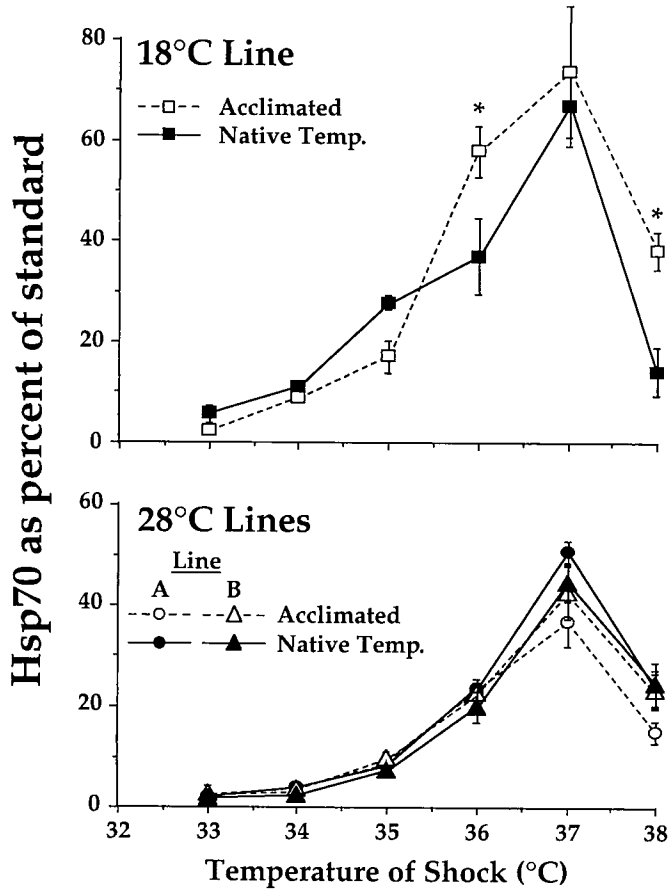

FIG. 1. Hsp70 levels (measured relative to a standard) induced by 1-h heat shocks (x axis) in third-instar larvae of the 18°C and replicate 28°C lines, following one generation at 25°C (open symbols) or when taken from native rearing temperature (shaded symbols). Asterisks indicate a significant effect of acclimation at individual temperatures ($P < 0.005$, Bonferroni-Dunn test). Symbols are means ± 1 SE.

Bonferroni-Dunn tests for line effect, $P \gg 0.005$ at each temperature).

Thermotolerance

The experimental lines have evolved differences in both basal and inducible thermotolerance (Fig. 3). Basal thermotolerance of 38.5°C varied significantly among the lines (ANOVA on line, df = 4, $F = 8.585$, $P < 0.0001$); both 25°C lines had the lowest mean survival. Inducible thermotolerance displayed the opposite relationship: survival of 38.5°C and 39°C (following 1 h at 36°C/1 h at 25°C) was lowest in the 28°C lines. This variation is significant in both

TABLE 2. Analysis of variance of effect on Hsp70 expression of heat-shock temperature (temp) and experimental line (line).

Source	df	SS	MS	*F*-ratio	Prob > *F*
Line	4	3332.594	833.149	5.644	0.0002
Temp	5	80,106.469	16,021.294	108.532	<0.0001
Line × temp	20	7619.853	380.993	2.581	0.0004
Residuals	209	30,852.124	147.618		

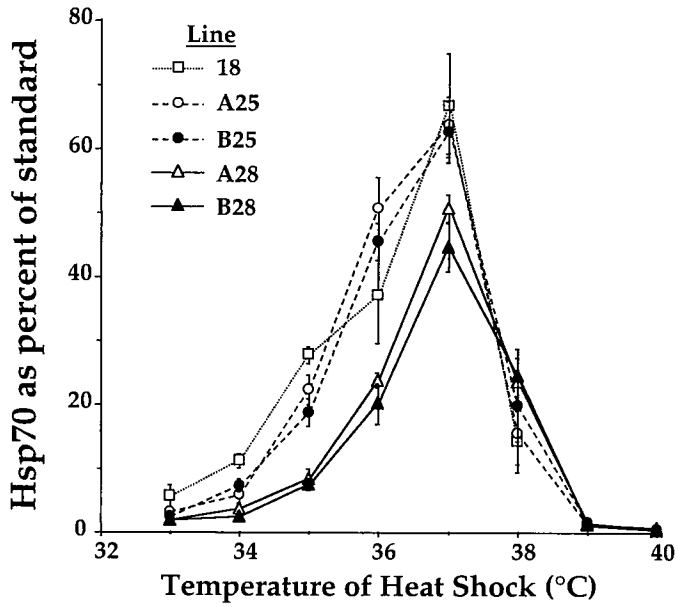

FIG. 2. Hsp70 levels (measured relative to a standard) in third-instar larvae following 1-h heat shocks (x axis). Larvae were collected from their native rearing temperature (18°C, 25°C, or 28°C). Replicate lines share symbol and hashing type. Symbols are means ± 1 SE.

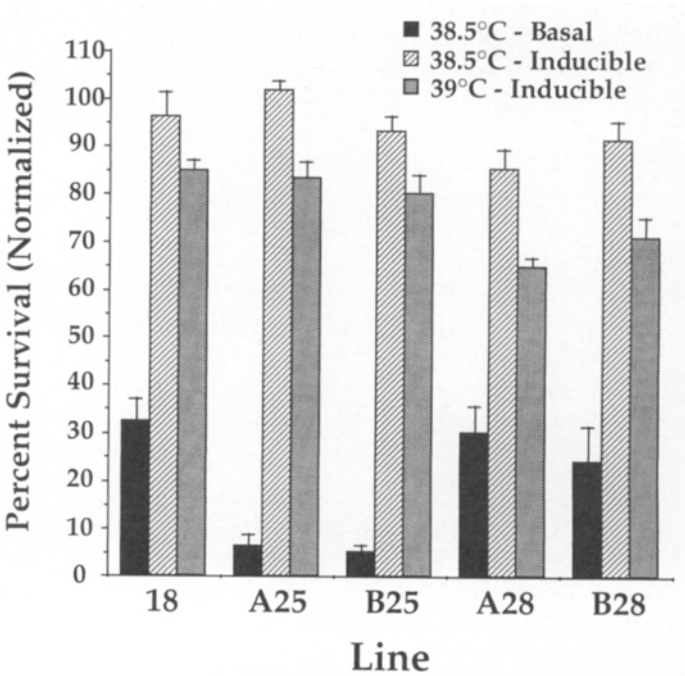

FIG. 3. Basal and inducible thermotolerance (scored as survival to adult stage, normalized to 25°C control groups) of third-instar larvae. Black bars indicate basal tolerance of 38.5°C (survival of 1 h at 38.5°C without pretreatment). All lines had zero basal tolerance of 39°C. Hashed bars indicate inducible tolerance of 38.5°C and 39°C (survival after 1 h pretreatment at 36°C, a 1 h recovery at 25°C, and then 1 h at 38.5°C or 39°C). Development was at 25°C before and after treatment. Values are means ± 1 SE.

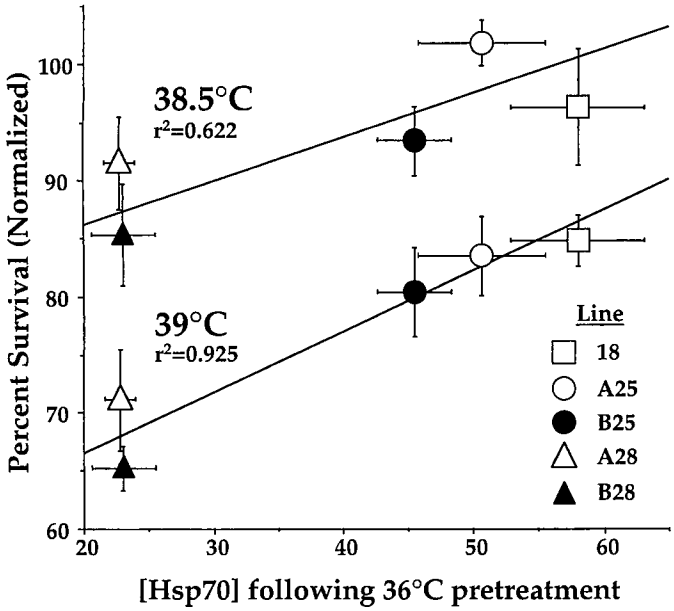

FIG. 4. The relationship between Hsp70 expression following 1 h at 36°C and inducible tolerance of 38.5 and 39°C (1 h at 36°C, 1 h at 25°C, 1 h at 38.5/39°C). Plotted are RMA regressions of line mean survival (normalized to 25°C control groups) on line mean Hsp70 expression. Symbols are survival means ± 1 SE (x direction: Hsp70 level; Y direction: survival).

cases, highly so at 39°C (ANOVA on line, df = 4, $F = 2.765$, $P = 0.04$ at 38.5°C; df = 4, $F = 5.811$, $P = 0.0004$ at 39°C). All lines had zero basal thermotolerance of 39°C.

Inducible thermotolerance is positively correlated with Hsp70 expression (Fig. 4). Reduced major axis (RMA) regressions of line mean survival of 38.5°C and 39°C on line mean Hsp70 expression at 36°C (the experimental pretreatment) are highly significant in both cases (38.5°C: $r^2 = 0.622$, $y = 78.702 + 0.37457x$, $P = 0.0009$; 39°C: $r^2 = 0.925$, $y = 56.025 + 0.52426x$, $P < 0.0001$). Larvae underwent one generation at 25°C before analysis of thermotolerance; thus, expression means used in this regression are those of 25°C-acclimated (one generation) larvae.

Hsp70 Copy Number

Five *hsp70* copies are in the genome of each experimental line (Fig. 5). In all five lines, an *hsp70*-specific DNA probe hybridizes to five distinct bands in Southern blots of bulk genomic DNA (Fig. 5C). The bands are of the sizes expected for a BamHI/BglI digest (Fig. 5B; Holmgren et al. 1979). The same probe hybridizes to four bands in *Drosophila simulans* DNA (Fig. 5C, lane 1), and with greater intensity for transgenic three- and six-extra *hsp70* copy lines (Fig. 5C, lanes 7 and 8). Additionally, PCR with *hsp70Bb*- and *hsp70Bc*-specific primers successfully amplified both genes (Fig. 5A) in more than 32 individual flies from each line (data not shown), discounting polymorphism within bulk DNA samples used for Southern blotting.

DISCUSSION

Evolutionary and Acclimatory Change in Hsp70 Expression

Throughout the history of life on earth, the stress-inducible Hsp70 genes have been notoriously refractory to evolutionary modification of their sequence, structure, regulation, and the function of the proteins they encode (see Introduction). Accordingly, that laboratory natural selection at different temperatures can alter Hsp70 expression curves in the experimental lines is surprising. Moreover, our laboratory study shows that change in temperature alone is sufficient for Hsp70 expression levels to evolve quickly and thereby extends several recent analyses of natural variation in Hsp70 expression (diIorio et al. 1996; Krebs and Feder 1997a).

Experimental evolution altered both the magnitude and thermal sensitivity of Hsp70 expression in the experimental lines. The reduction in magnitude is striking: at 34–37°C, the 28°C lines express 30–50% less Hsp70 than the other lines. For Hsp70 expression after heat shocks of 36° or less, the variation among the laboratory lines was "compensatory" (Hazel and Prosser 1974), with the lines evolving at lower temperatures expressing more Hsp70 at lower heat-shock temperatures. By contrast, for Hsp70 expression after heat shocks of > 37°C, the 28°C lines expressed no more Hsp70 than the other lines; experimental evolution at high temperature did not right-shift the 28°C lines' expression curves to higher inducing temperatures.

The variation in expression among the experimental lines is within the range of natural variation (Krebs and Feder 1997a), which is not unexpected. Laboratory evolution generally does not alter traits to the same extremes as commonly seen in artificial selection experiments (Rose et al. 1996). Alternatively, the 28°C lines may have exhausted genetic variation for Hsp70 expression, requiring new mutations for further change in expression. Also, selection on other physiological or cellular factors (e.g., increases in overall stability of non-Hsp proteins or change in the autoregulation of Hsp70 expression) could yield the pattern we found. For example, because thermal denaturation can induce Hsp70 expression, greater protein thermal stability in the 28°C lines could reduce Hsp70 induction upon heat shock.

Our examination of phenotypic plasticity of Hsp70 expression involved acclimating lines to a new temperature for one generation. Previous studies of other traits (Cavicchi et al. 1995) acclimated these lines for four generations before analysis to eliminate possible cross-generational effects (see Huey et al. 1995). However, four generations may be sufficient for Hsp70 expression to decline in wild *D. melanogaster* after establishment in laboratory culture (R. Krebs, pers. comm.). We therefore chose one generation of acclimation, a compromise intended to remove most maternal and environmental effects without introducing potentially confounding genetic changes. In this design, acclimation to 25°C had little impact on Hsp70 expression in the 28°C lines, which suggests that differences among these lines (see above) are nonplastic and evolutionary. This outcome is not universal; in other organisms, 70-kD Hsp expression curves changed with season (Fader et al. 1994; Roberts et al. 1997), time of day (Colombo et al. 1995), and short-term laboratory acclimation (Koban et al. 1991; Dietz 1994). These other studies

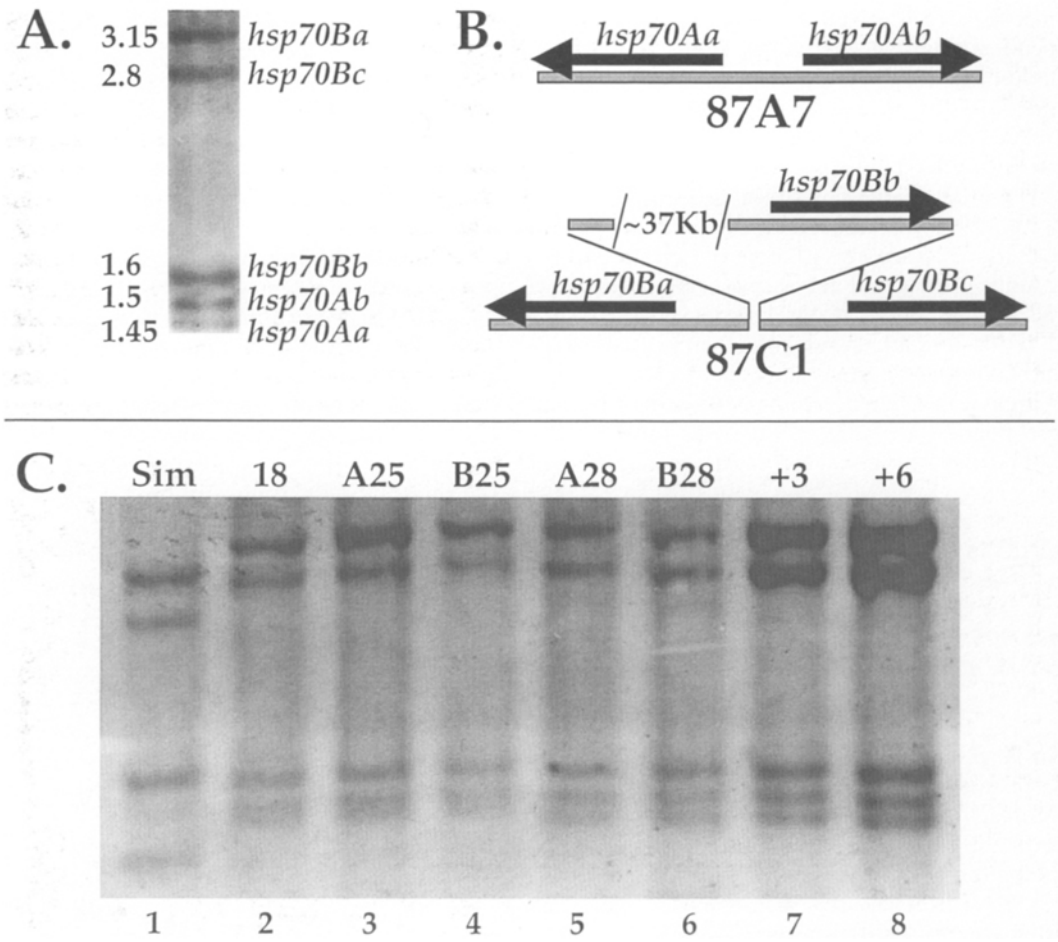

FIG. 5. Number and organization of *hsp70* genes. (A) Sizes (in kb) and derivation of fragments. (B) Schematic of *hsp70* genomic location, organization, and nomenclature (modified from Leigh Brown and Ish-Horowicz 1981). (C) Southern blot of bulk genomic DNA samples, digested with BamHI and BglI, hybridized with *hsp70*-specific probe. Lane 1, *D. simulans*; lanes 2–6, experimental lines; lanes 7 and 8, transgenic lines with three and six extra *hsp70* genes, respectively (Welte et al. 1993).

are not strictly comparable with ours, however. Due to technical limitations, they treated the entire family of 70-kD Hsps, typically comprising many different proteins, as a single variable. Our work, on the other hand, exploited a highly specific antibody to quantify a single protein species.

Thermotolerance

Numerous molecular, cellular, and physiological processes underlie thermotolerance (Moseley 1994), which suggests its evolutionary change would require modification of several genes or processes. The various Hsps figure prominently in this list, with their number and diversity further contributing to potential genetic redundancy. However, experimental manipulation of specific *hsp70* loci causes large modification of thermotolerance in many organisms and cell types (see table 1 in Feder and Hofmann 1999), even though they typically express large and diverse suites of heat-inducible Hsps. By contrast, *D. melanogaster* expresses only a handful (most prominent are Hsp83, Hsp70, and Hsp26/27), and Hsp70 is by far the predominant protein species (Lindquist 1984). Together with *hsp70*'s amplification at the genomic level (Leigh Brown and Ish-Horowicz 1981), extremely high expression level (Krebs and Feder 1997b), and tight experimental link to inducible thermotolerance (Solomon et al. 1991; Li et al. 1995; Feder et al. 1996), evidence suggests that *Drosophila* exploits Hsp70 in thermotolerance to an extent unparalleled in other species. Thus, it is perhaps not surprising that modification of Hsp70 in *Drosophila*, even at the small scale of experimental evolution, could cause a significant effect on thermotolerance.

Experimental evolution altered Hsp70 expression and thermotolerance in concert in our experimental lines: the 28°C lines both express less Hsp70 and have lower inducible thermotolerance of 38.5°C and 39°C than do the other lines. The high degree of correlation between Hsp70 expression and thermotolerance (see Fig. 4) supports the hypothesis that differences in the expression of a single gene can have a large physiological impact in evolution (Feder 1996). In the 25°C lines, experimental evolution lowered basal thermotolerance (relative to the other lines), but Hsp70 expression and inducible thermotolerance were high. This pattern is consistent with two hypotheses concerning the distinction of inducible versus basal thermotolerance in *D. melanogaster*. First, Hsp70 may play a key role in the former but not necessarily

the latter. Second, the two traits may be decoupled and free to evolve independently (Boon-Niermeijer et al. 1986).

Given the relationship of Hsp70 to thermotolerance in *Drosophila*, the lower Hsp70 expression in the 28°C line would at first seem maladaptive. Low Hsp70 expression could be advantageous in this line, however. At the cellular level, Hsp70 expression in the absence of stress is detrimental to growth (Feder et al. 1992). In transgenic *Drosophila* larvae, overexpression of Hsp70 initially provides greater thermotolerance, but inhibits growth and decreases survival to adulthood (Krebs and Feder 1997b). Natural variation in Hsp70 expression and larval-to-adult survival in benign environments are similarly correlated (Krebs and Feder 1997a). Thus, the benefits of high Hsp70 expression may trade off with deleterious consequences in nature. In our 28°C lines, these opposed stabilizing forces are unbalanced. At constant 28°C, high Hsp70 levels may never be advantageous because heat shock may never be a problem. However, development is extremely rapid at this temperature; delay or perturbation would be deleterious. Thus, even the small amount of Hsp70 expressed below 32°C (in cells: Velazquez et al. 1983; O'Brien and Lis 1991; brain: Krebs and Feder 1997c; muscles: Wheeler et al. 1995) could negatively affect fitness: selection would favor reduction in expression in the 28°C lines.

Our results for larvae generally agree with earlier findings for adults in these lines, which vary in both inducible and basal thermotolerance (Cavicchi et al. 1995). When each line is challenged with the same temperatures, larval and adult inducible thermotolerance are inversely proportional to rearing temperature. We ascribe the few discrepancies between ours and the earlier studies to differences in specifics of heating regime, developmental stage, and the relative robustness of the lines. For example, Cavicchi et al. (1995) used a "sliding scale" of heat shock temperatures, shocking 28°C adults at very high temperatures. In that study, 28°C adults lacked inducible thermotolerance at more than 41°C. However, 28°C larvae exhibited inducible thermotolerance at 38.5°C and 39°C. Additionally, basal thermotolerance is directly proportional to culture temperature in adults (Cavicchi et al. 1995), whereas no such relationship exists in larvae. Stage-specific differences in thermotolerance are not uncommon: In *Drosophila buzzatii*, artificial selection for adult thermotolerance did not yield a correlated increase in larvae or vice versa (Loeschcke and Krebs 1996). Additionally, the overall poor health of the 18°C flies (lower fecundity and survival, see Methods and Materials) may be mostly manifested in adult traits, and thus cause lower adult thermotolerance in this stage.

Molecular Bases of Variation in Hsp70 Expression

Despite anecdotal reports of *hsp70* copy number polymorphism within the ancestral Oregon R strain (Ish-Horowicz et al. 1979a), we find no evidence of such variation among or within the experimental lines. Southern blotting and locus-specific PCR confirm that *hsp70* copy number was unaffected by experimental evolution. Whether copy number varies in or among other lines remains to be seen. However, copy number can evolve rapidly in a phylogenetic context: *D. melanogaster* is unique in having five *hsp70* genes. The primitive condition of the genus (and all dipterans, Benedict et al. 1993) is two copies in an inverted pair arrangement. Within the *melanogaster* subgroup, this two-copy cassette is duplicated: *Drosophila yakuba, teisseri, mauritiana*, and *simulans* all have four *hsp70* genes (Leigh Brown and Ish-Horowicz 1981). Only in *melanogaster* is a pair disrupted and a single gene tandemly duplicated. We are currently comparing *hsp70* sequences and copy number in the *melanogaster* group to shed light on the evolutionary history of these loci. For example, a lack of sequence variation in the *hsp70Bb-hsp70Bc* region would indicate that this tandem duplication may actually be fixed in *D. melanogaster*.

Given the detailed sequence and functional information available for *hsp70*, determining the molecular bases of evolutionary variation in expression should be tractable. In our experimental lines, transcriptional differences at least partially account for differences in Hsp70 expression. *Hsp70* mRNA level after heat shocks of 32–44°C varies in a pattern congruent with Hsp70 protein level, that is, 28°C adults transcribe the least *hsp70* at most temperatures (Cavicchi, unpubl. data), potentially because *cis*-regulatory regions differ. Indeed, selection for high knockdown temperature changes frequency of *hsp68* promoter alleles (McColl et al. 1996); we are currently screening each *hsp70* promoter for variation in conserved promoter sequence elements critical for inducible expression. Alternatively, variation in the *hsp70* 5′- and 3′-untranslated regions, which affect message stability (Petersen and Lindquist 1989; Hess and Duncan 1996), and numerous *trans*-acting factors such as HSF, the heat shock transcription factor, may underlie evolutionary modification of Hsp70 expression.

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