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Muscle-specific expression of *Drosophila* hsp70 in response to aging and oxidative stress

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ABSTRACT **Induction of *Drosophila* hsp70 protein was detected during aging in flight muscle and leg muscle in the absence of heat shock, using an hsp70-specific monoclonal antibody, and in transgenic flies containing hsp70–β-galactosidase fusion protein reporter constructs. While hsp70 and reporter proteins were induced during aging, *hsp70* message levels were not, indicating that aging-specific induction is primarily posttranscriptional. In contrast, *hsp22* and *hsp23* were found to be induced during aging at the RNA level and with a broader tissue distribution. The same muscle-specific hsp70 reporter expression pattern was observed in young flies mutant for catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6). In catalase (*cat*) hypomorphic lines where flies survived to older ages, the time course of hsp70 reporter expression during aging was accelerated, and the initial and ultimate levels of expression were increased. The hsp70 reporter was also induced in young flies mutant for copper/zinc superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1). Taken together, the results suggest that aging-specific hsp70 expression may be a result of oxidative damage.**

During aging virtually every *Drosophila* organ system that has been examined exhibits decreased function and degenerative changes at the tissue and cell level (1). Aging-associated changes occur at the molecular level as well, because organisms ranging from nematodes to flies to humans have been shown to accumulate inactive or altered proteins, such as enzymes that are partially denatured, oxidized, and catalytically inactive (2–4).

Oxidative damage to proteins can result from reaction with oxygen radicals formed as a by-product of normal metabolism. Cellular defenses against oxygen radicals include the enzymes copper/zinc superoxide dismutase (Cu/ZnSOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) and catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6). Cu/ZnSOD converts the free radical superoxide to hydrogen peroxide, and catalase converts hydrogen peroxide to water and molecular oxygen. *Drosophila* homozygous for mutations in either the Cu/ZnSOD or catalase (*cat*) genes exhibit increased sensitivity to oxidative stress and have reduced viability and longevity (5–7). Flies that overexpress Cu/ZnSOD, alone or in combination with overexpression of catalase, exhibit increased resistance to some forms of oxidative stress and are reported to have increased longevity (8, 9).

When abnormal or denatured proteins are produced in cells by various experimental treatments, it results in an induction of the heat shock proteins (hsps) (10–13). Studies of hsps in *Drosophila* and other organisms suggest that hsps function to promote protein renaturation, prevent further protein aggregation and denaturation, and facilitate proteolytic pathways (14, 15). Induction of the heat shock genes by heat shock has been studied in detail and in *Drosophila* utilizes both transcriptional and posttranscriptional controls (16, 17).

Here we report that in *Drosophila* the highly conserved hsp70 is also induced in response to aging.

MATERIALS AND METHODS

***Drosophila* Culture.** Oregon-R strain flies were grown on cornmeal/agar medium (18). Flies were cultured at 25°C until 0–2 days posteclosion, and then male flies were transferred to 29°C. Male adults were maintained at 50 per vial and transferred to fresh vials every 4 days. Fifty percent survived to age 26 days, 15–20% survived to 37 days, and no flies survived beyond 47 days (data not shown). Transgenic fly stocks are homozygous for the chromosome bearing the *P*-element insertion and have decreased longevity; at 29°C, 50% survive to 14 days (see Fig. 4). "Young" refers to flies 5–7 days posteclosion, and "old" refers to flies 36–38 days posteclosion. Young flies were heat shocked at 37°C for 2 hr (17).

Drosophila Stocks. *Drosophila* strains are as described (19). The lines transgenic for heat shock gene promoter–β-galactosidase (β-gal) fusion constructs are Bg61 (20) and −194(84E) (17), and both contain *hsp70* sequences −194 to +1011. *cat* point mutations and deficiency stock are as described (refs. 5 and 6; obtained from J. W. Mahaffey). Cu/ZnSOD null mutation and deficiency stock are as described (ref. 7; obtained from J. P. Phillips).

Western Analysis. SDS/PAGE was according to standard procedures (21). Protein was transferred to nitrocellulose and incubated with hsp70-specific rat monoclonal IgG 7FB (22). Primary antibody binding was detected with alkaline phosphatase-conjugated mouse anti-rat IgG (Boehringer Mannheim). Total protein extracted from young and old thoraces was ≈70 μg per thorax. Data were quantitated by scanning filters with an Apple Macintosh OneScanner and using OFOTO image analysis software (Apple Computer, Cupertino, CA). Alternatively, Western analysis was performed using the monoclonal antibody MA3-007 (Affinity Bioreagents, Neshanic Station, NJ), which recognizes both the *hsp70* and the heat shock 70 cognate (*hsc70*) gene products.

RNA Analysis. For Northern blot analysis, a synchronized population of 2000 Oregon-R male flies was maintained at 29°C, and at time points 50 flies were dissected into head, thorax, and abdomen, and total RNA was isolated. In certain experiments, samples were first normalized to rRNA content as determined by a preliminary Northern blot. Identical results were obtained with and without such normalization.

The hsp probes were gene-specific fragments from the *hsp22*, *−23*, *−26*, and *-27* genes (23) and the entire *hsp70* gene (24). Control probes were alcohol dehydrogenase (Adh) (25), β-tubulin (26), ribosomal protein 49 (rp49) (27), esterase (28), apurinic endonuclease 3 (AP3) (29), and eIF-4A (30). To detect *hsp70* RNA specifically, the probe was the unique *hsp70* 5′ leader sequences −194 to +260 (31).

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Abbreviations: Cu/ZnSOD, copper/zinc superoxide dismutase; hsp, heat shock protein; hsc, heat shock cognate; β-gal, β-galactosidase; rp49, ribosomal protein 49; Adh, alcohol dehydrogenase.

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Reverse transcription–PCR analysis was by standard methods (32). For *hsc3* (33), the upstream primer was 5′-TGCCGAGAAGTTCGCCGATGAGGACAAG-3′ (+1904 to +1932), and the downstream primer was 5′-CATCGCCGCCCTCTGGAGGAGGAGCACC-3′ (+2218 to +2191). For *hsp70* (24), the upstream primer was 5′-CAATTCAAACAAGCAAAGTGAACACGTC-3′ (+5 to +33), and the downstream primer was 5′-GTGAGTTCTTCTTTCTCGGTAACTTGTT-3′ (+119 to +91). Downstream primers were used for reverse transcription of the *hsp70* and *hsc3* RNAs simultaneously. Upstream primers were ^{32}P-labeled with polynucleotide kinase, and products were visualized by autoradiography. PCR product was a linear function of input reverse transcription product. The identity of the reverse transcription–PCR products was confirmed by size and by restriction digestion. Results were quantitated by densitometry.

Spectrophotometric Assay of β-gal Activity. β-gal enzymatic activity was quantitated in tissue extracts using published procedures (31). Assays were performed under conditions where the reaction was linear with regard to the amount of extract. Data are presented as the average of duplicate samples or the average ± SD for triplicate samples.

***In Situ* Staining for β-gal Activity.** β-gal expression was visualized in dissected flies and cryostat sections using published procedures (17).

RESULTS

hsp70 Protein Expression Is Induced in the Thorax During Aging. Western analysis was performed on total protein extracted from young (5–7 day) and old (36–38 day) male heads, thoraces, and abdomens. hsp70 was detected using monoclonal antibody 7FB, which recognizes only the heat-inducible form of hsp70 and does not recognize the constitutively expressed hsp70-related family members, the heat shock 70 cognates (hscs) (22). hsp70 protein was found to be induced 7- to 10-fold in the thorax of old flies, relative to young flies (Fig. 1*A*, compare lanes 1 and 2 to lanes 3–5, and additional data not shown), whereas no increase was observed in head or abdomen (Fig. 1*A*, lanes 10–17). Induction was indeed specific to the true hsp70 protein, as total hsc70 family protein levels detected with monoclonal antibody MA3-007 were not induced (Fig. 1*B*).

Heat Shock Gene RNA Levels During Aging. Northern analysis was performed on RNA from head, thorax, and abdomen of flies at time points throughout the adult life-span. Blots were hybridized with probes specific for several heat shock genes and for several non-hsp controls (Fig. 2). Most heat shock genes exhibited an accumulation of RNA at day 2 after eclosion, which decreased with age.

Strikingly, an increase in RNA was observed for the *hsp22* and *hsp23* genes in the thorax at the later time points, particularly 40 days. When corrected relative to rp49 for loading differences, this increased expression was 8- to 10-fold for *hsp22* and 4- to 8-fold for *hsp23* between days 20 and 40. Induction was specific for these heat shock genes, as the control and other *hsp* transcripts did not exhibit the same pattern of accumulation. Adh and β-tubulin RNA levels appear to be slightly reduced relative to rp49, AP3, esterase, and eIF-4A at 40 days. In independent experiments, a dramatic increase in *hsp22* RNA levels was observed at 30 and 40 days in the head and abdomen as well as the thorax (Fig. 2*B*), and the same result was observed for *hsp23* (data not shown). *hsp22* and *hsp23* induction is more obvious in this experiment apparently because expression at earlier time points is decreased. The reason for this variability in expression at early time points is currently unknown but may be greater when flies are cultured at higher density. For *hsp26* and *hsp27* probes, no significant increase in RNA levels was detected (≤2-fold).

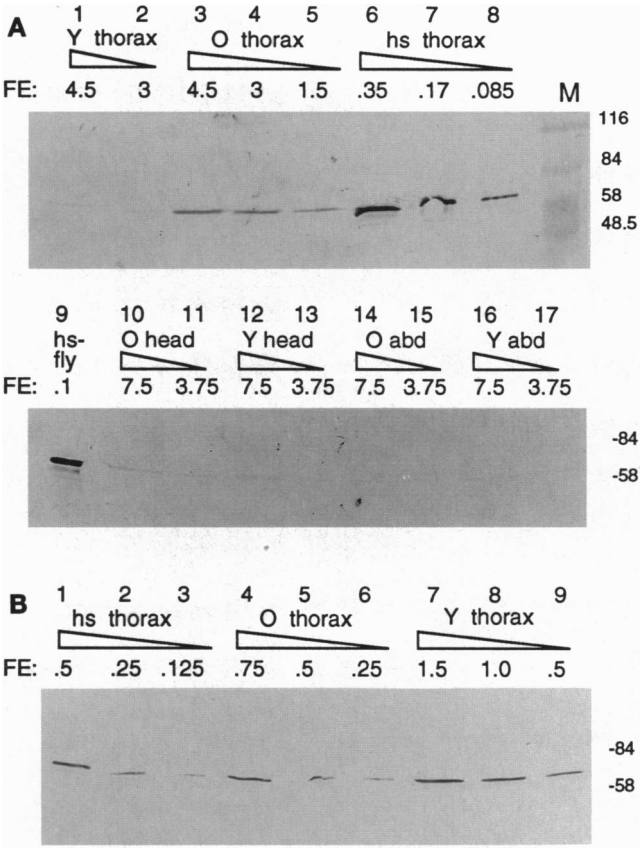

FIG. 1. Western analysis of hsp70 protein induction during aging. Total protein was isolated from heads, thoraces, and abdomens of young (Y), old (O), and heat-shocked (hs) flies. FE, fly equivalents. (*A*) Hsp70 detected using monoclonal antibody 7FB. The alkaline phosphatase reaction was developed slightly longer for the head and abdomen (abd) samples to reveal the trace signal that does not change with age. (*B*) Total hsc70 protein detected using monoclonal antibody MA3-007. M, molecular size markers in kDa.

The *hsp70* genes are highly homologous to the constitutively expressed *hsc70* genes (33). To assay *hsp70* RNA specifically, three approaches were used. The first was Northern blot analysis using diverged *hsp70* 5′ leader sequences as a probe (Fig. 2*C*). Relative to the rp49 control, the *hsp70* RNA levels are approximately constant with age. In the second approach, quantitative reverse transcription–PCR was performed using primers homologous to unique sequences in the *hsp70* 5′-leader region, and again no increase in *hsp70* RNA levels was detected (Fig. 2*D*). The *hsc3* gene was used as a constitutively expressed control (Fig. 2*D*). Thus, *hsp70* RNA levels do not detectably increase with age, and therefore we conclude that the observed increase in hsp70 protein levels is due to post-transcriptional regulation. This conclusion was confirmed by the third approach of Northern analysis of the marked *hsp70* transgenes described below.

hsp70–β-gal Reporter Fusions Are Induced During Aging. Young and old transgenic flies containing the hsp70 protein-coding region fused to *Escherichia coli* β-gal were assayed for β-gal (17, 20, 31). A 7- to 10-fold increase in β-gal activity in the thorax was observed during aging (Table 1). We have recently obtained similar results with multiple transgenic lines containing *hsp70* sequences −194 to +260 (amino acid residue 7) fused to the β-gal gene (J.C.W. and J.T., unpublished data). No significant induction of β-gal activity was detected in heads or abdomens using this spectrophotometric assay, whereas dramatic induction of β-gal was detected in all samples after heat shock (Table 1). Transgene transcripts were quantitated by Northern blot analysis using a β-gal-specific probe and were

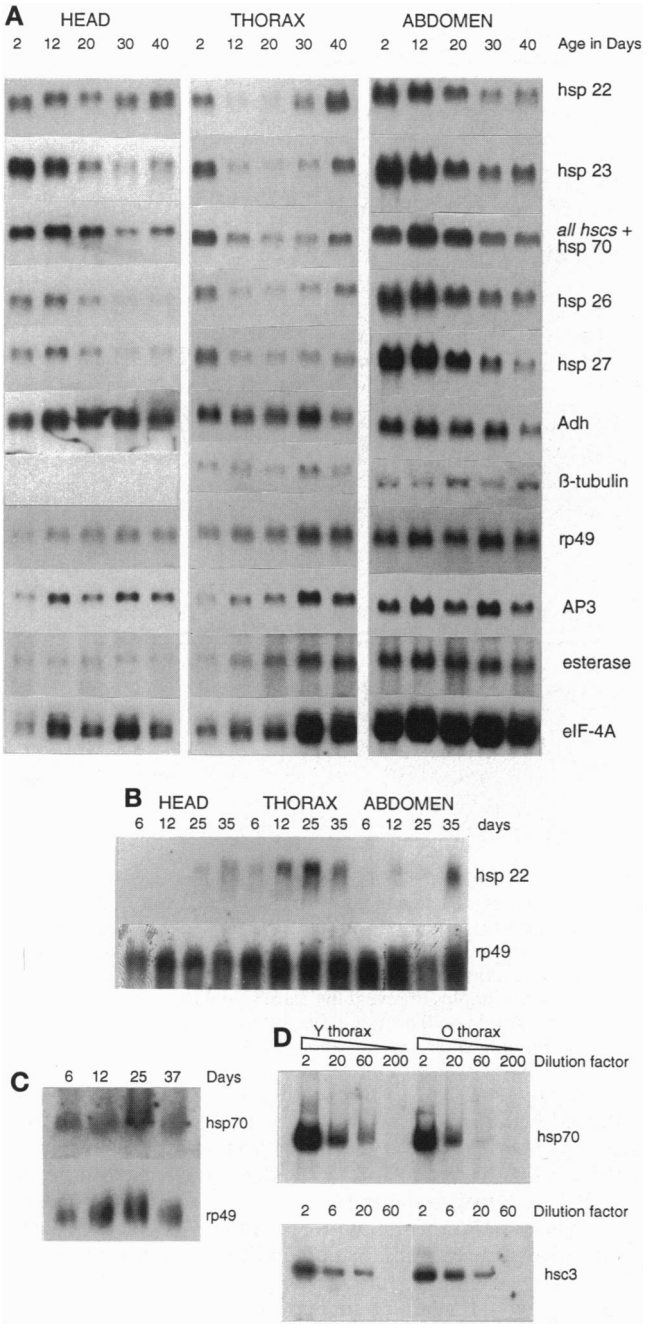

FIG. 2. RNA analysis of heat shock gene expression during aging. (*A*) Northern blot analysis. Total RNA was Northern blotted and analyzed with the indicated probes. (ß-tubulin was not assayed for the head.) (*B*) Northern analysis as in *A*, with independent RNA samples. (*C*) Northern analysis of thorax RNA. (*D*) Quantitative reverse transcription–PCR analysis. The indicated dilutions of reverse-transcribed thorax RNA were analyzed. No signal was obtained without reverse transcriptase (data not shown). Y, young; O, old.

equal or slightly reduced in old flies relative to rp49 (data not shown). Thus both endogenous and transgenic *hsp70* genes exhibit posttranscriptional upregulation during aging.

***hsp70* Expression During Aging Is Specific to the Flight Muscles and Leg Muscles.** The tissue specificity of hsp70–β-gal expression was examined further by staining for β-gal activity *in situ* (Fig. 3). No hsp70–β-gal staining was observed in young flies (Fig. 3*A*). In old flies hsp70–β-gal expression was observed specifically in the indirect flight muscles (Fig. 3*B*), the direct flight muscles (data not shown), and in the muscles of the legs (Fig. 3*C*). hsp70–β-gal expression was not detected in any other tissues at this level of analysis (Fig. 3, and additional data not shown).

Table 1. hsp70/β-gal fusions with heat shock and aging

Line name	Body segment	β-gal activity			Fold induction	
		Young	H.S.	Old	H.S.	Old
−194 (84E)	Head	0.013	0.44	0.024	34	1.8
	Thorax	0.070	2.07	0.502	30	7.2
	Abdomen	0.123	1.49	0.077	12	0.63
Bg61	Thorax	0.018	0.70	0.191	39	10.6

H.S., heat shocked.

The Time Course of hsp70–β-gal Expression Is Proportional to Life-Span. Higher culture temperatures decrease *Drosophila* life-span and proportionally accelerate the time course of deteriorative changes (34, 35). hsp70–β-gal reporter expression was assayed in flies cultured at 29°C, 25°C, and 18°C and was observed to be delayed at lower temperatures, corresponding to the increased life-span (Fig. 4). When flies cultured at lower temperatures were maintained for long enough periods, hsp70–β-gal reporter expression reached nearly the same levels as in flies cultured at 29°C (data not shown).

***hsp70* Reporter Expression in *cat* and Cu/ZnSOD Mutant Flies.** We tested the effect of reduced oxygen radical defenses on *hsp70* expression. The *"Bg61" hsp70*–β-gal reporter was placed by recombination onto the same chromosome as a small deletion uncovering the *cat* gene, $df(3L)Cat^{DH104}$. Crosses were performed to generate flies heterozygous for this *"Bg61 dfCAT"* chromosome and different *cat* alleles of varying severity (6). The control was flies heterozygous for the *Bg61 dfCAT* chromosome and the nonmutant parental chromosome used to originally generate the *cat* mutations (*C*). *cat* alleles *N1* and *N4* exhibit no detectable catalase enzymatic activity in tissue extracts (6). *N4* lacks the initiator AUG and produces no detectable protein, whereas *N1* has only a single amino acid substitution in the heme-binding domain (5, 6). *cat* mutant flies of genotype *Bg61 dfCAT/N4* and *Bg61 dfCAT/N1* were found to exhibit high levels of hsp70–β-gal reporter expression, even in young flies (Fig. 5*A*). *Bg61 dfCAT/N4* flies had greatly reduced viability and did not survive beyond ≈5 days. However, *Bg61 dfCAT/N1* flies survived to older ages, presumably due to some residual function of the N1 mutant protein, and in these flies reporter expression increased with age with a faster time course and to higher levels than in control flies containing *Bg61 dfCAT/C* (Fig. 5*A*). *cat* alleles *N3* and *N5* each have ≈4.5% of wild-type levels of catalase enzymatic activity in tissue extracts. *Bg61 dfCAT/N5* flies exhibited increased hsp70–β-gal expression relative to control, which appeared to increase with age, whereas *Bg61 dfCAT/N3* showed little difference from control (Fig. 5*B*). Again, the catalase activity detectable in extracts may not exactly correlate with activity *in vivo*. *cat* allele *N2* has ≈12% of wild-type activity, and the time course for *Bg61 dfCAT/N2* was indistinguishable from control (data not shown). In all cases, the hsp70–β-gal expression was specific for the flight muscles and leg muscles at all ages. This apparent threshold of catalase activity sufficient for a time course of hsp70–β-gal expression equal to the control is consistent with previous reports that catalase activity is present in excess (6).

An analogous experiment was performed with the Cu/ZnSOD gene. The same *Bg61 hsp70*–β-gal reporter was placed by recombination onto a chromosome bearing a small deficiency that uncovers the Cu/ZnSOD locus, *df(3L)h-76*. This *"Bg61 dfSOD"* chromosome stock was then crossed to flies containing the Cu/ZnSOD null allele SOD^{n108} (7), to generate flies of genotype *Bg61* $dfSOD/SOD^{n108}$. Because of the reduced viability and life-span of these Cu/ZnSOD mutants,

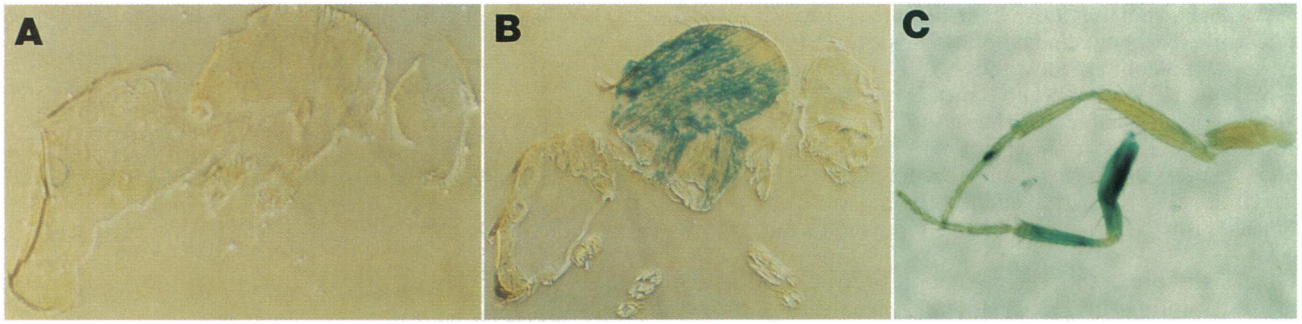

FIG. 3. hsp70–β-gal reporter expression *in situ*. (*A*) Young males of line −194(84E) were sectioned and stained for β-gal. Head (anterior) is to the right. (*B*) Old males of line −194(84E). The blue-stained tissue is indirect flight muscle. Muscles of each leg were also darkly stained essentially identical to the pattern seen in *C*, and identical results were obtained with line Bg61 (data not shown). When Bg61 and −194(84E) lines are subjected to heat shock, every tissue is intensely stained (ref. 20; data not shown). (*C*) Four-day-old males of genotype *Bg61 dfCAT/catalase*^{N4} and control genotype *Bg61 dfCAT/C* dissected and stained for β-gal. The top, unstained leg is the control, and the lower is the *cat* mutant, in which the leg muscles are darkly stained. Intense staining was also present in the direct and indirect flight muscles, as in *B* (data not shown).

flies could not be analyzed beyond ≈3 days. These flies were found to exhibit high levels of hsp70–β-gal reporter expression even at 1 day (Fig. 5*A*), again preferentially in the flight muscles and leg muscles. However, in the Cu/ZnSOD mutant flies, some induction was also observed in head tissues. The same induction and staining patterns were obtained when the experiment was performed using a different hsp70–β-gal reporter, −194(84E) (data not shown).

DISCUSSION

The induction of *hsp70* during aging in *Drosophila* reported here is specific for flight muscle and leg muscle. This is quite distinct from the ubiquitous pattern of expression observed in response to heat shock (20). In flies null or hypomorphic for either Cu/ZnSOD or *cat*, hsp70–β-gal reporter expression was observed in young flies, with the same preference for flight muscle and leg muscle, and under appropriate conditions showed an accelerated increase with age. The simplest explanation of these data is that the flight muscle and leg muscle expression of *hsp70* during normal aging and in Cu/ZnSOD and *cat* mutant flies occurs through the same pathway and is a consequence of oxidative damage. We note that previous studies did not detect *hsp70* expression in young or old *Drosophila* in the absence of heat shock (36). We conclude that this difference in results reflects the greater sensitivity of the assays employed in this study.

The flight muscle and leg muscle specificity of hsp70 expression during aging may result because these tissues are preferentially susceptible to some type of damage or alteration taking place. A second possibility is that the relevant damage is taking place throughout the organism, but hsp70 expression is a tissue-specific response to this damage, for which a pathway exists only in flight muscle and leg muscle.

The posttranscriptional upregulation of hsp70 protein expression and the increase in *hsp22* and *hsp23* RNA levels during aging are significantly different from the well-characterized response of the *hsp* genes to a heat shock. *hsp70* RNA was found to be expressed at a relatively low, constitutive

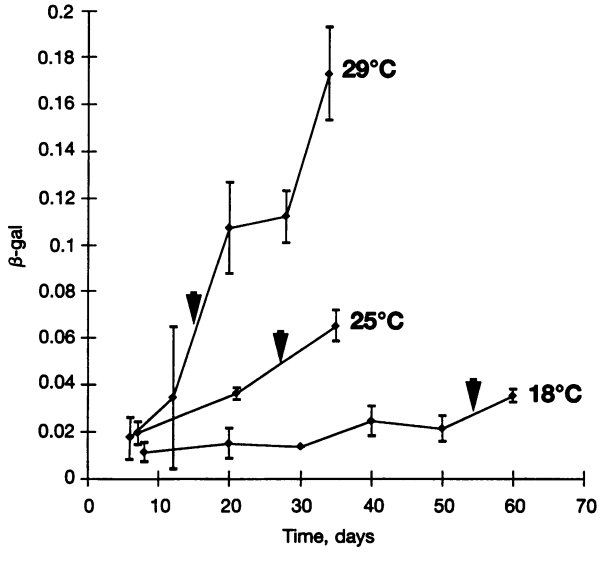

FIG. 4. hsp70–β-gal reporter expression in flies cultured at different temperatures. β-gal was quantitated in extracts of thoraces for line −194(84E) cultured at 29°C, 25°C, and 18°C. Fifty percent survival times (≥200 flies assayed) are indicated with arrows. Data points are the average ± SD of triplicate assays. Analogous results were obtained with line Bg61 (data not shown).

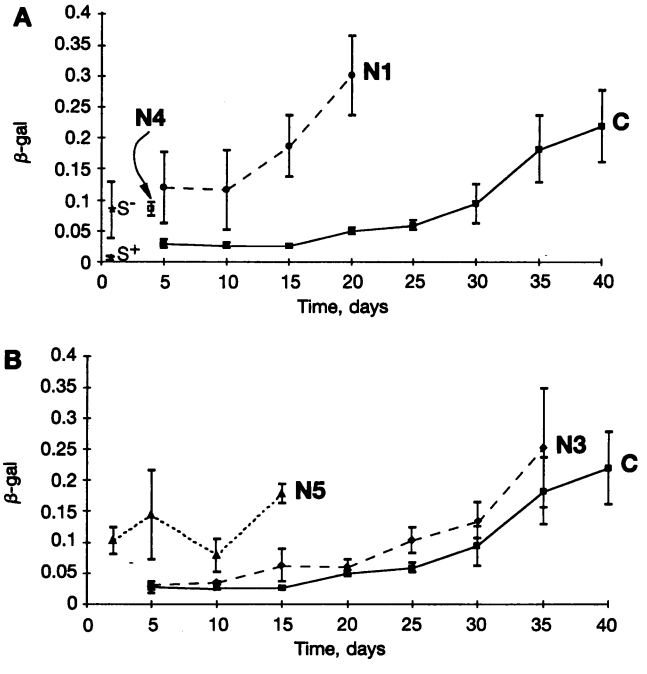

FIG. 5. hsp70–β-gal reporter expression in *cat* and Cu/ZnSOD mutants. β-gal was quantitated in extracts of thoraces. Control flies (C) were of genotype *Bg61 dfCAT/C*. The experimentals (*N1–5*) were of genotype *Bg61 dfCAT/N*, where *N* is the particular *cat* allele. Cu/ZnSOD control flies (S^+) were of genotype *Bg61dfSOD/C*. Cu/ZnSOD mutant flies (S^-) were of genotype *Bg61 dfSOD/SOD*^{N108}. Data points are the average ± SD of triplicate assays. The data is presented in two graphs for clarity.

level, which had not previously been detected for *hsp70*. In fact, our recent experiments indicate that this expression requires previously unidentified constitutive *hsp70* promoter elements (J.C.W. and J.T., unpublished data). hsp70 induction during heat shock involves dramatic transcriptional and translational upregulation. The posttranscriptional upregulation of hsp70 protein expression during aging occurs in the absence of transcriptional upregulation and may be the same or a different mechanism than the translational upregulation during heat shock.

Finally, the preferential upregulation of *hsp22* and *hsp23* RNA levels during aging must also be mechanistically distinct from their induction during heat shock, since during heat shock these genes exhibit a smaller degree of RNA level upregulation than the other *hsp* genes, such as *hsp70*.

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