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Genetics

Drosophila drop-dead mutations accelerate the time course of age-related markers

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ABSTRACT **Mutations of the *drop-dead* gene in *Drosophila melanogaster* lead to striking early death of the adult animal. At different times, after emergence from the pupa, individual flies begin to stagger and, shortly thereafter, die. Anatomical examination reveals gross neuropathological lesions in the brain. The life span of flies mutant for the *drop-dead* gene is four to five times shorter than for normal adults. That raises the question whether loss of the normal gene product might set into motion a series of events typical of the normal aging process. We used molecular markers, the expression patterns of which, in normal flies, change with age in a manner that correlates with life span. In the *drop-dead* mutant, there is an acceleration in the temporal pattern of expression of these age-related markers.**

Shortened life span has been observed due to various single gene mutations in both humans and flies. In *Drosophila*, the mutations *Hyperkinetic*^{1} (*Hk*^{1}) and *Shaker*^{5} (*Sh*^{5}), which affect nervous system activity (1), as well as mutations affecting antioxidant systems (catalase, superoxide dismutase), lead to shortened life span, thought to result from effects on energy metabolism and/or oxidative stress (2–8). In humans, the progeroid syndromes, such as Werner and Hutchison–Guilford, may be considered dramatic examples of rapid aging, although not all organ systems are involved. The shortened life span in Werner is about a factor of two; in Hutchison–Guilford it is of the same order as in *drop-dead* (9).

It has been shown that various genes are expressed in the adult fly in characteristic, reproducible, temporal patterns throughout its adult life (10–12). When the life span of the adult is altered, either by temperature or genetic means, the relative temporal patterns of expression of certain of these genes remain the same, but on a time scale changed in proportion to life span (10, 11). These expression patterns thus would appear to relate more closely to physiological than chronological age, providing a set of molecular biomarkers of the rate of "aging."

If early death in the *drop-dead* (13–15) animal is due to a defect that abruptly interrupts the normal sequence of age-related gene expression, the temporal pattern of the molecular biomarkers should be initially normal, but prematurely truncated. If, however, mutations in the *drop-dead* gene result in acceleration of the normal scenario, the usual sequence of gene expression should occur, but proceed more rapidly. In this report, we demonstrate that, in *drop-dead*, the temporal patterns of expression of three different molecular biomarkers are accelerated.

MATERIALS AND METHODS

Fly Strains and Culture Conditions. The *drop-dead*^{x3} allele isolated by Buchanan and Benzer (15) was used for the experiments described in this paper. Similar results were found with the *drop-dead*^{1} allele (13). *wingless* (*wg*) and *engrailed* (*en*) enhancer-trap stocks were obtained from J. Kassis (16). The 206 enhancer trap line, obtained from Tim Tully, contains a *LacZ*-expressing construct (17). Control flies were Canton-S-5, which is a stock homozygous for an X chromosome derived from a Canton-S strain and an FM7 balancer stock, as described in (18). All studies were done with F_1 offspring from crosses in which virgin females of the phenotype *drop-dead*^{x3}/FM7c or Canton-S-5 were mated to males carrying the *wg*, *en*, or 206 enhancer-trap chromosome; each F_1 male examined possessed one copy of the *wg*, or the *en*, or the 206 enhancer-trap chromosome.

All flies were kept in plastic vials containing a standard cornmeal agar medium, with several grains of yeast added (19). Approximately 30 flies initially were in each vial; survivors were passed to fresh vials every 7 days. All flies were cultured in humidified, temperature-controlled environmental chambers at 25°C throughout development. Adult flies were collected, without anesthesia, within 2 hr of emergence from the pupal case. For the experiments using *wg* and *en*, flies were then placed in humidified, temperature-controlled environmental chambers set at 25°C . For 206, 18°C was used because of the rapidity of the rising curve of expression of that marker. Male and female flies were aged together in the same vials.

Life Span Analysis. Life span studies were carried out using the methods of collection and culture noted above, except that the flies were passed into fresh vials every other day, at which time the numbers of dead males were recorded. Over 400 male flies were scored for each life span study. Life spans were defined as times to 1% survival, which were 14 and 50 days, respectively, for *drop-dead wg* and control *wg* animals, 14 and 60 days for *drop-dead en* and control *en* animals, and 35 and 150 days for *drop-dead* 206 and control 206 animals. The scaling factors for the three markers were 3.6, 4.3, and 4.2, respectively, for *wg*, *en*, and 206. Some differences may possibly be due to secondary variations in genetic background.

Whole-Mount 5-Bromo-4-Chloro-3-Indolyl β-D-Galactoside (X-Gal) Staining. For each time point, at least 10 male fly heads were removed, fixed for 20 min in 1% glutaraldehyde in phosphate buffered saline (1 × PBS), and reacted with a standard X-Gal solution (GIBCO/BRL) (19). X-Gal reactions were performed at 37°C, 18 hr for the *wg* and 206 enhancer-trap lines, and 2 hr for the *en* line, which has a higher level of expression of β-galactosidase (β-gal) (10). After the X-Gal reaction, heads were rinsed twice with 1× PBS and stored in 70% glycerol in 1× PBS. The antennae were cut off and placed under coverslips on microscope slides. Video images or photographs were taken with a ×16 Leitz objective.

Quantitation of β-Gal Expression in Whole-Mount Adult Antennae. Rather than the standard assay (20), we used the more sensitive X-Gal method to measure the amount of β-gal expressed in the combined third and fifth segments of the

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Abbreviations: *wg*, wingless; *en*, engrailed; β-gal; β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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antenna. An optically based, computer-assisted video microscopy system that integrates the blue color into arbitrary units was used to quantify the X-Gal reaction (10, 21). Antennae from at least 10 animals were sampled at each time point, except for days 9 and 10 in the case of *drop-dead wg* (where only 2–4 antennae were available, see Fig. 2), and days 4 and 9 in *drop-dead en* (6 and 4 antennae, respectively, see Fig. 3). The reliability of the video microscopy system is such that repeated capture of the same image shows <2% variation.

RESULTS

Life Span of *drop-dead* Animals. Fig. 1*A* shows survival curves for *drop-dead* mutant flies, compared with normal controls. While *drop-dead* flies live about five times shorter, the shapes of the survival curves are similar. As is true for normal flies (22), death in *drop-dead* occurs more rapidly at higher ambient temperature (Fig. 1*B*).

Age-Related Markers. We chose the three *lacZ*-marked (17, 23–25) genes *wg* (16), *en* (16), and 206 (10, 26), whose relative temporal patterns of expression of β-gal have been shown to correlate with life span at different ambient temperatures (18°C, 25°C, and 29°C). *wg* has previously been tested both vs. ambient temperature and in combination with the reduced life span mutants Hk^1, Sh^5, and *superoxide dismutase* (SOD^{N1}) (ref. 27 and B.R. and S.L.H., unpublished data). Genes *en* and 206 have so far been tested only vs. ambient temperature (ref. 27 and B.R. and S.L.H., unpublished data). In this paper, we examined all three in the background of the *drop-dead* mutation. Expression of β-gal was measured in the antenna as a function of chronological age at 25°C and at 18°C, using the method of Helfand *et al.* (10) based on the optical absorption of the blue X-Gal reaction product. Since experiments have shown that the enzyme decays within a few hours (26) this is a valid measure of current synthesis of β-gal.

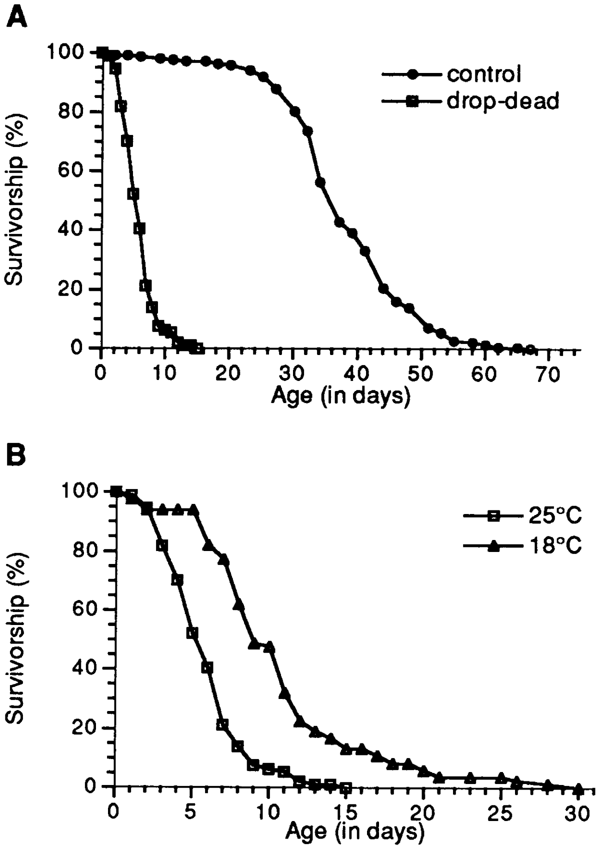

FIG. 1. Effect of *drop-dead* on life span. (*A*) Survival curves of adult male *drop-dead* and control flies at 25°C. (*B*) *drop-dead* males at 25°C vs. 18°C. All animals in this figure contain one autosomal copy of the *en* enhancer-trap P-element insert, with or without *drop-dead* on the X-chromosome.

Acceleration of Age-Related Markers in *drop-dead* Animals. *Effect of* drop-dead *on* wg *expression.* The enhancer-trap line marking the *wg* gene normally shows a level of β-gal expression that declines from the time of eclosion, reaching a low value at around days 10–15 at 25°C (Fig. 2*A*) (ref. 27 and B.R. and S.L.H., unpublished data). Since the β-gal expression occurs in a sizable proportion of the antennal cells, and examination of serial sections revealed no decrease in cell count (26), it is unlikely that the decline can be attributed to cell degeneration. In a *drop-dead* background, the decline is accelerated, reaching its lowest level of expression by days 3–4 (Fig. 2 *A* and *B*). In

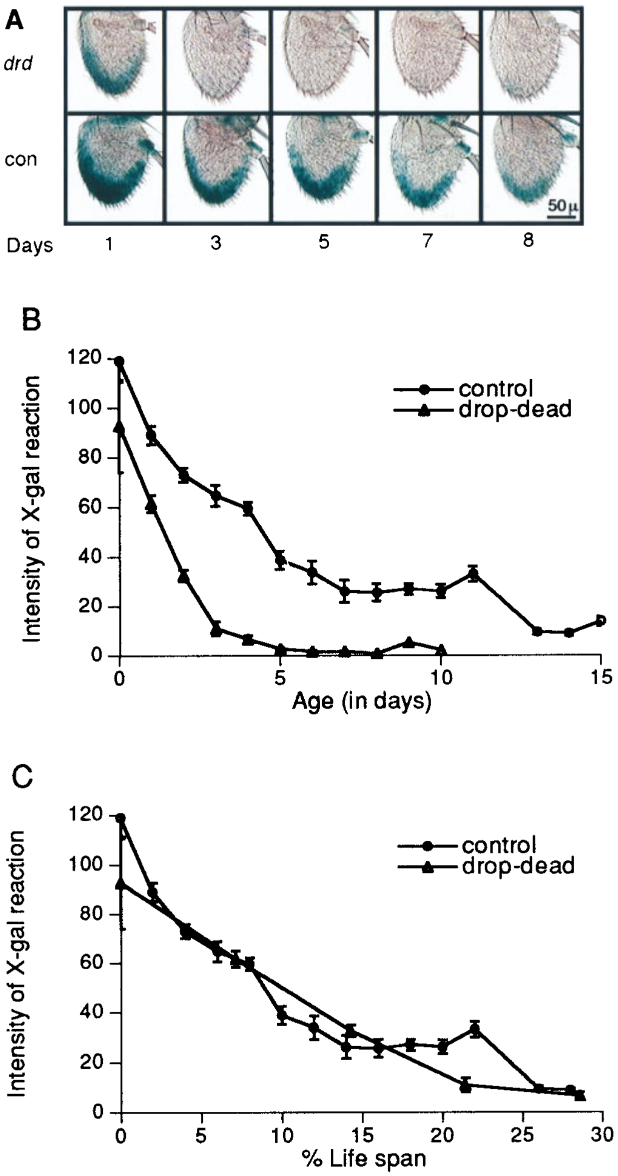

FIG. 2. Temporal patterns of expression of the *wg* gene in the antennae of adult *drop-dead* vs. control males. (*A*) Photomicrographs of whole-mount adult antennae from animals containing one autosomal copy of the *wg* enhancer-trap chromosome, with or without *drop-dead* on the X-chromosome, reacted with X-Gal to reveal blue staining in cells expressing β-gal. Each is a typical example from over 10 flies at each age after emergence of the adult. (*B*) Relative amount of β-gal expressed in the combined third and fifth antennal segments of *drop-dead* vs. control males at 25°C, plotted against chronological age. (*C*) Plotted against percent life span. (Bars = SEM.)

Fig. 2*C*, where the data are replotted as percentage of life span rather than calendar time, the curves closely resemble one another.

Effect of drop-dead *on* en *expression.* In the enhancer-trap line marking the *en* gene, β-gal expression in the antenna shows a complex temporal pattern characterized by oscillations superimposed on a general decline (27). In a *drop-dead* background, the pattern is similar, but accelerated (Fig. 3*A*). The temporal pattern of expression, normally extending over a period of some 2 months in the *lacZ* strain at 25°C, is compressed into the 2 weeks of life in the presence of the *drop-dead* mutation (Fig. 3*B*).

Effect of drop-dead *on 206 expression.* The third marker used was an enhancer-trap strain, designated 206 (10, 26), in which the amount of β-gal expression in the antenna normally increases rapidly in the first days of adult life. This rise was missed in a previous study (26) in which the earliest time point taken was at 10 days, at which time a plateau had already been reached. Because of the relatively fast time scale of expression of this marker, the assay of the effect of *drop-dead* was performed at 18°C to slow down the time course. This marker is especially pertinent since its increase with time counteracts possible concern that the β-gal-expressing cells might be degenerating with age. In the *drop-dead* background, β-gal expression in 206 was already strong on day 1, a plateau being attained by day 3 (Fig. 4*B*). At 18°C, the maximal life span of *drop-dead* in a 206 background at 18°C was about 40 days, as compared with about 155 days for 206 in the absence of the *drop-dead* mutation. When plotted with respect to percentage of life span, the temporal pattern of β-gal expression of the 206 marker in the *drop-dead* animals was comparable with the control animals (Fig. 4*C*).

DISCUSSION

The finding that the temporal expression patterns of three different biomarkers, including one that rises with age, roughly scale in proportion to the life span of the *drop-dead* animals, suggests that, during its shortened life, the *drop-dead* fly undergoes, more rapidly, a series of events normally associated with advancing age.

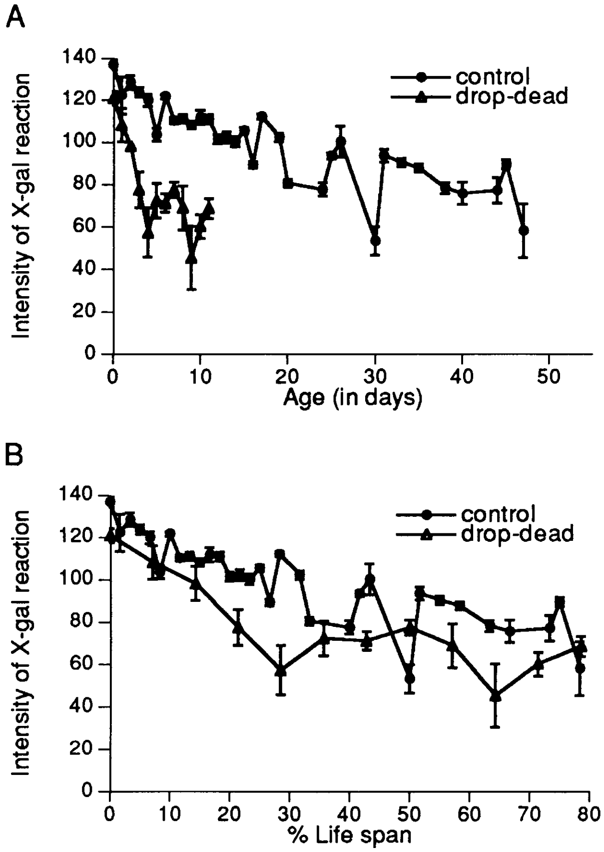

FIG. 3. Temporal patterns of expression of the *en* gene in the antennae of *drop-dead* vs. control males at 25°C. (*A*) Plotted against chronological age. (*B*) Plotted against percent life span. Each animal contained one autosomal copy of the *en* enhancer-trap chromosome, with or without *drop-dead* on the X-chromosome. (Bars = SEM.) Photographs not shown because the high level of expression in *en* makes it difficult to see differences in photographs that are clearly seen using the computer-assisted optical system.

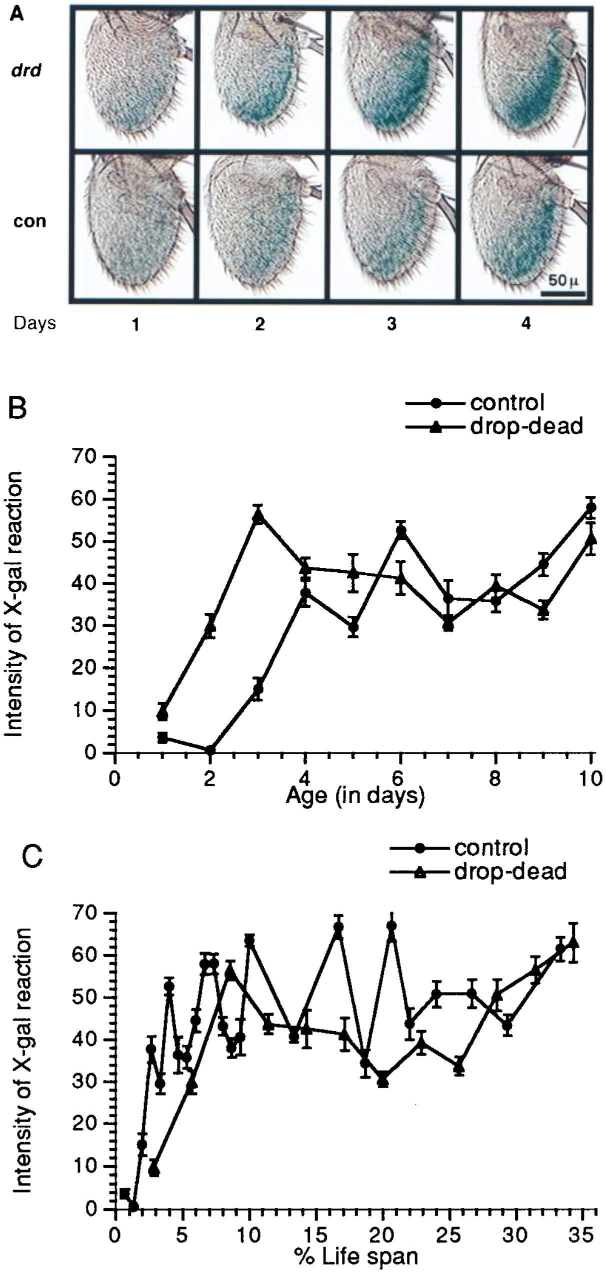

FIG. 4. Temporal patterns of expression of the 206 enhancer trap-marked gene in the antennae of *drop-dead* vs. control males at 18°C. (*A*) Photomicrographs of whole-mount adult antennae reacted with X-Gal. Each is a typical example from over 10 flies at each age. (*B*) Relative amount of β-gal expressed in the antennae, plotted vs. chronological age. (*C*) Plotted vs. percent life span. Each animal contained one autosomal copy of the 206 enhancer-trap insertion, with or without *drop-dead* on the X-chromosome. (Bars = SEM.)

It is important to note that such acceleration of the age-associated scenario by mutations in the *drop-dead* gene does not identify the normal gene as a longevity gene, *per se. Hyperkinetic*^{1} (*Hk*^{1}), *Shaker*^{5} (*Sh*^{5}), and *SOD*^{N1} have similar effects on accelerating the choreography of the age-related markers (refs. 11 and 27 and B.R. and S.L.H., unpublished data), yet they all involve different physiological effects. *Hk*^{1} is defective in a beta subunit of the potassium channel (28), *Sh*^{5} is defective in an alpha subunit of the potassium channel (29–31), and *SOD*^{N1} is defective in an enzyme (superoxide dismutase) (8). These clearly cannot all be "the aging gene," yet mutations in all of them appear to speed up ongoing processes that, in normal flies, proceed with chronological time and culminate in death of the fly. It remains to identify the key physiological events in the scenario illuminated by the changing expression patterns of the marker genes.

Note, that in the search for genes conferring longevity, one of the difficulties is the long time one must wait to score survival. The rapidity of *drop-dead's* demise offers the advantage that it can facilitate the search for such genes (Y.-J. Lin and S.B., unpublished data).

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