Timing of Expression of a Gene in the Adult Drosophila Is Regulated by Mechanisms Independent of Temperature and Metabolic Rate

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ABSTRACT

The examination of β-galactosidase (β-gal) expression in the third segment of the antenna of the 2216 enhancer trap line in Drosophila melanogaster reveals two distinct spatial and temporal regulatory patterns of expression during adult life. Type I expression is characterized by a decline in the level of β-gal expression with increasing age. Starting from a maximal level of expression at the time of adult emergence, there is a decrease in the number of cells that express β-gal so that by 40–50 days of adult life few cells express β-gal. Varying the ambient temperature and using hyperactivity mutants (Hyperkinetic, Shaker) demonstrates that the rate of this decline is independent of temperature and metabolic rate. Type II expression is distinctly different in spatial distribution and temporal regulation from the first pattern. Type II expression is restricted in the antenna to a small (<20–30) set of cells whose level of expression changes in a periodic manner with time. The regulation of this periodicity appears to be linked to ambient temperature.

For D. melanogaster, the timing of age-related changes appears to be coupled to the physiological and/or oxidative state of the animal. A variety of observations have demonstrated a close relationship between the metabolic rate and oxidative state of the fly and the timing of age-related events: (1) inverse relationship of temperature and life span in poikilothermic animals reviewed in MIQUEL et al. (1976), (2) genetic mutations that alter physical activity, rate of oxygen consumption and life span in D. melanogaster (TROUT and KAPLAN 1970), (3) effect of physical restriction on the life span of houseflies (Sohal and BUCHAN 1981), and (4) relationship between oxidative state and longevity (ORR and SOHAL 1994; DUDAS and ARKING 1995, SOHAL et al. 1995).

The finding of genes whose temporal pattern of expression is linked to physiological age provides further support for the relationship between the metabolic rate and oxidative state and rate of aging in adult D. melanogaster (Helfand et al. 1995; Rogina and Helfand 1995; Wheeler et al. 1995). In these studies it was shown that many genes in the adult D. melanogaster show characteristic changes in the level of expression of an associated reporter protein during adult life. These changes in the level of expression are nonrandom and define a temporally driven pattern of expression. For those genes examined, alterations in the metabolic rate of flies, by mutations (e.g., Hyperkinetic, Shaker) or by varying ambient temperature, results in compensatory changes in the temporal pattern of expression. These changes appear to be linked to life span and suggest that these genes are regulated by physiological age not calendar time. One of the issues that has been raised
by these studies is whether the regulation of expression of all such reporter gene marked genes whose pattern of expression changes during adult life is linked to ambient temperature and metabolic rate.

In this report we show our findings on a gene in *D. melanogaster* whose timing of expression over the first 40–50 days of adult life appears to be regulated independently of temperature and metabolic rate. The temporal pattern of expression of this gene appears to follow calendar time/chronological age rather than physiological age.

**MATERIALS AND METHODS**

**Fly stocks:** The description of the 2216 enhancer trap line can be found in Helfand et al. (1995). Genetic analysis has shown that the 2216 gene is on the second chromosome (S. L. Helfand, unpublished results). *Hyperkinetic* (Hk) and *Shaker* (Sh) stocks were obtained from the Drosophila Stock Center in Bloomington Indiana. Animals used for the studies with Hk and Sh possess one copy of the 2216 enhancer trap marked second chromosome. Animals in all other studies were homozygous for the 2216 enhancer trap marked second chromosome. The studies with Hk and Sh were done with F1 offspring from crosses in which virgin females homozygous for Hk, Sh, or wild type (Canton-S and Oregon-R) were mated to males homozygous for the 2216 enhancer trap containing second chromosome. Males hemizygous for the Hk and Sh mutations exhibit the anesthesia-induced “shaking” phenotype, while females heterozygous for either of these two mutations appear wild type and do not show the anesthesia-induced shaking phenotype (Lindsley and Zimm 1992).

**Fly culturing:** All flies were kept in plastic vials containing a standard corn-meal agar medium with several grains of yeast added (Ashburner 1989). Approximately 30 flies were in each vial; flies were passed to fresh vials every 7 days. All flies were cultured in humidified temperature-controlled environmental chambers at 25° throughout development. Adult flies were collected without anesthesia within 2 hr of emergence from the pupal case and put in humidified temperature-controlled environmental chambers set at 18°, 29° or 25°.

**Life span:** Life span studies were carried out using methods of collection and culture noted above except that the flies were passed into fresh vials every other day at which time the number of dead males and females were recorded. Over 400 of collection and culture noted above except that the flies exhibit the anesthesia-induced “shaking” phenotype. The reliability of this method of collection and culture noted above except that the flies exhibit the anesthesia-induced “shaking” phenotype.

**Quantitation of β-gal expression in whole mount adult antenna:** The amount of β-gal expressed in the antennae of the 2216 enhancer trap line is too low to be detected by standard chromogenic assays such as chlorophenol red-β-D-galactopyranoside (CPRG) (Glaser and Lis 1990). For the quantification of β-gal, we made use of an optically based computer-assisted video microscopy system that uses X-gal to detect β-gal expression in whole mounts and has been detailed previously (Blake et al. 1995; Helfand et al. 1995). At least 20 antennae were sampled at each time point, using methods previously described (Blake et al. 1995; Helfand et al. 1995). Standard procedures for X-gal staining were followed (Ashburner 1989; Blake et al. 1995). The reliability of the video microscopy system is such that capture of the same image at different times shows <2% difference in values.

**RESULTS**

**Spatial distribution shows two distinct patterns of expression with the 2216 enhancer trap line:** The lack of cell division (Bozuck 1972; Ito and Hotta 1992), the persistence of most cells in the third segment of the antennae throughout life (Helfand and NapRta 1996) and the availability of molecular markers such as enhancer traps, which allow the visualization of expression of single genes at the level of individual cells, highlight the reasons for using *D. melanogaster* as a model for examining gene expression during aging (Helfand et al. 1995; Rogina and Helfand 1995).

The enhancer trap line 2216 was used to examine the spatial pattern of expression of this gene in the antenna during the life span of the adult. The 2216 lacZ reporter is expressed in two distinct patterns in adults. The type I pattern is characterized by the expression of the reporter protein β-gal in many of the cells of the third segment of the adult antenna. From this broad pattern, the number of the cells expressing the reporter declines over a period of 30–40 days. The decline occurs in a characteristic spatial pattern with the cells continuing to express the reporter consolidating at the proximal portion of the third segment (see Figure 1A).

Type II pattern of expression is distinguished by a high level of expression in a small number of cells, <20–30, distributed in a characteristic spatial pattern throughout the third segment (Figure 1B). This pattern of expression is best seen at a time following the complete or nearly complete loss of expression associated with the type I pattern (after day 40 in homozygotes, after day 5 in heterozygotes), although it appears that the type II pattern of expression is present throughout most of adult life.

Examination of the temporal pattern of expression in type II subset of cells reveals another interesting feature in the regulation of the reporter. Expression in these cells is not constant but appears to follow a well-regulated periodic oscillation, with expression in all or most of these cells changing in concert. Expression is present, absent, and then present again in these cells over a period of several days. For example, Figure 1B shows a representative antenna grown at 25° and stained with X-gal showing no staining on day 45 but a full complement of 20–30 cells stained on day 46. Staining in these cells then declines to nearly undetectable levels by day 49 and returns to near maximal intensity of expression on day 51. This oscillation between maximal and undetectable levels of expression can be seen whenever it is not masked by the more diffuse type I pattern of expression (for homozygotes after day 40 until the end of life, for heterozygotes after day 5 until the end of life.)

Quantitative analysis of the type I pattern of 2216 expression shows a temporal pattern of regulation: Us-
FIGURE 1.—Photomicrographs of whole mount adult antennae from enhancer trap line 2216 at different ages reacted with X-gal to reveal blue staining in the nuclei of cells that are expressing β-gal. Each photograph represents a typical example from over 60 (A) or 30 (B) different antennae examined for each time point. Days listed on the bottom are from the time of the adult’s emergence from the pupal case. (A) Expression of β-gal over the first portion of the adult’s life span. (B) A higher magnification view of antennae from the period between 45–51 days.

ing a computer-assisted video system, we determined the level of β-gal in the third segment of the antenna from the 2216 enhancer trap line throughout adult life. As seen in Figure 2, the most prominent feature in the temporal pattern of expression is a decline in the amount of β-gal from the time of eclosion. The decline in the level of gene expression appears to follow a nearly linear decay, although subtle periodic oscillations may be noted after day 5. It is likely that these periodic oscillations in the level of β-gal expression are due to the periodic type II pattern of expression that is superimposed upon the overall declining expression of β-gal associated with the type I pattern of expression. The decline in the type I pattern of expression reaches its lowest point at or near zero between 40–50 days of age in males (Figure 2). The expression of β-gal after 40–50 days is due only to the type II pattern of expression. In females there is generally a lower level of expression and the point at which there is little to no staining is seen a little earlier than in males, at ~35–40 days of adult life (data not shown).

Alteration of the rate of aging and life span using ambient temperature shows that the timing of the expression of the type I pattern of 2216 gene expression is independent of temperature: We have recently shown that the regulation of a number of genes in the adult *D. melanogaster* is linked to physiological age and not chronologic age/calendar time (Helfand et al. 1995; Rogina and Helfand 1995). Since *D. melanogaster* are true poikilotherms, ambient temperature can be used to alter the rate of aging and life span of the animal (Figure 3). We measured the level of expression of β-gal
throughout the life span of animals from the 2216 enhancer trap line that had been raised as adults at 18° or 29° to gain insight into the mechanisms of regulation of the 2216 gene in the adult animal. The type I pattern of expression of the 2216 gene for adults at either 29° or 18° follows a decline from the time of eclosion that is similar to the pattern seen at 25° (Figure 4, A and B).

Despite the differences in the absolute levels of expression (expression at 29° > 25° > 18°), the calendar time in days at which the level of expression of the 2216 gene approaches zero for the type I pattern of expression tends toward 40–50 days regardless of the temperature at which the adult is kept. This can be directly seen for those animals raised at 18° (Figure 4B) but must be extrapolated for those animals raised at 29° (Figure 4A). Since the maximum life span of the adult animal at 29° is ~41 days, it is difficult to obtain sufficient numbers of animals for our quantitative analysis after day 30. However, a linear extrapolation of the trend established by the points available at 29° lead to an intersection on the X-axis between 40–50 days. Therefore, despite an 11 degree difference in temperature, resulting in a nearly threefold difference in life span (Figure 3), the course of gene expression appears to be similar, terminating at approximately the same calendar time (between 40–50 days). Furthermore, if the level of expression for each of the points is normalized to the level of expression on day 1 for each of the temperatures, then the points for each of the three temperatures nearly superimpose (Figure 5A). The temporal regulation of the type I pattern of expression of the 2216 gene appears to be unaffected by ambient temperature.

Figure 5B illustrates that the type I pattern of expression is more closely associated with calendar time/chronological age than physiological age. In this figure the level of expression for each of the three different ambient temperatures is plotted against the percentage of life span of the animal, a rough measure of physiological age. For genes whose expression is linked to physiological age, plots of gene expression vs. percentage of life span converge and are very similar at different temperatures (Helfand et al. 1995; Rogina and Helfand 1995). In the case of 2216, however, the point at which the type I level of expression approaches zero is ~40–50% of the life span for animals living at 18°, ~70% of the life span for animals living at 25°, and nearly 100% of the life span for animals living at 29°.

In addition, superimposed upon the decline of β-gal expression are periodic oscillations that appear to be due to β-gal expression contributed by the type II pattern. This is best seen at 18° when the absolute level of β-gal associated with the type I pattern decreases, while the level of β-gal expression in cells associated with the type II pattern continues to be expressed at a high level. This results in the more readily observable oscillations at 18° as opposed to 25° and 29°.

Alteration of the rate of aging and life span using hyperactive mutants shows that the timing of type I gene expression is independent of metabolic rate: The level of β-gal expressed was measured throughout the life span of animals in which the 2216 marked gene was placed into a background of the X-linked Hk1 or Sh5 mutations. Control crosses were performed using Canton-S and Oregon-R wild-type strains. Crosses were done so that the F1 males being tested were hemizygous for the Hk1 or Sh5 mutant chromosome and possess only one copy of the 2216 marked second chromosome. These F1 males have their X chromosome derived from either a Canton-S or Oregon-R wild-type strain. These F1 males were generated from similar crosses except that the parental females were either from a Canton-S or Oregon-R wild-type strain. The F1 male controls were generated from similar crosses except that the parental females were either from a Canton-S or Oregon-R wild-type strain. These F1 males have their X chromosome derived from either a Canton-S or Oregon-R wild-type strain. The F1 male controls were generated from similar crosses except that the parental females were either from a Canton-S or Oregon-R wild-type strain. These F1 males have their X chromosome derived from either a Canton-S or Oregon-R wild-type strain. The F1 male controls were generated from similar crosses except that the parental females were either from a Canton-S or Oregon-R wild-type strain. The F1 male controls were generated from similar crosses except that the parental females were either from a Canton-S or Oregon-R wild-type strain.
A Gene Regulation During Adult Life

**Figure 4.**—Amount of antennal β-gal expression from the 2216 line grown at 29° (A) and 18° (B) throughout adult life. Age (in days) is from the time of the adults' emergence from the pupal case. At least 20 different antennae were sampled for each point. Error bars, SEM.

don-S or Oregon-R background and possess only one copy of the 2216 marked second chromosome. The F1 males hemizygous for Hk' or Sh5 were shown to have the expected life shortening phenotype (Figure 5). Male F1 animals in the Hk' and Sh5 mutant background live a maximum of 47 and 58 days at 25° and 66 and 78 days at 18°, respectively, while the matched F1 males in the Canton-S wild-type background live a maximum of 75 days at 25° and 131 days at 18°. The F1 males in an Oregon-R wild-type background live a maximum 102 days at 25°. As can be seen in Table 1, the age in days at which the type I pattern of expression declines to undetectable is similar in all genetic backgrounds and for both temperatures, 18° and 25°.

Although there is a large decrease in the absolute level of β-gal and an earlier time of cessation of the type I pattern of 2216 expression, there is no effect of the hyperactive/life-shortening phenotype on the pattern of expression of the 2216 gene. If anything the type I pattern of expression appears to be detectable for a longer time in the Hk' and Sh5 mutant background than in the Canton-S or Oregon-R background, although these differences are probably not significant (Table 1). The decrease in the absolute level of β-gal signal in animals with only one copy of the 2216 enhancer trap-containing second chromosome is seen when 2216 is placed into either a Canton-S, Oregon-R, Hk', or Sh5 background. This is largely due to there

**Figure 5.**—Intensities of X-gal reactions. (A) Intensity of X-gal reaction is plotted as a percentage of that at day 1. The percentage of that at day 1 was determined by dividing the intensity of X-gal reaction for each point (from Figures 2 and 4) by the intensity of X-gal reaction on day 1 for each of the three temperature conditions (from Figures 2 and 4): 29° (○), 25° (□), and 18° (△). (B) Intensity of the X-gal reaction plotted against percentage life span. The percentage life span for each value was determined by dividing the day for each point (from Figures 2 and 4) by the maximum life span of line 2216 at that temperature (from Figure 3). The curves were fit to the points for each temperature 29° (○), 25° (□), and 18° (△) using a linear equation. The SEM are the same as those in Figures 2 and 4 but have been omitted here for ease in viewing the graphs.
being only a single copy of the lacZ gene, instead of two. However, because of this marked decrease in the amount of β-gal, an effect of the insertion of the enhancer element itself into the 2216 gene locus, resulting in an increase in expression of β-gal when homozygous, must also be considered. A molecular genetic analysis of the 2216 locus will need to be done.

Quantitative examination of the type II pattern of expression suggests a periodic pattern of oscillation that appears temperature dependent: The type II pattern of expression seen with the 2216 marked gene is quite distinct from type I. Type II expression can be defined by the appearance of a small group of cells in characteristic positions throughout the third segment of the antenna (Figure 1B). The type II pattern is masked when type I expression is high. Visual inspection suggested that β-gal appearance in these cells was not constant, but changed in a nonrandom periodic fashion (Figure 1B). Quantitative analysis confirmed that there is a periodicity to the expression of β-gal in these cells. The duration of the period of expression appears to be linked to the ambient temperature at which the animals are living. This is most easily seen when type I expression is near zero. For example, after day 40 the level of β-gal expression associated with the type II pattern shows a regular oscillatory pattern with a period approximating 3–5 days for animals growing at 25°C. Animals living at 18°C show a period of between 12 and 15 days (Figure 7). In animals with two copies of the 2216 enhancer trap-containing chromosome, evidence for the periodic oscillatory pattern of type II may be seen during the first 40 days despite the masking by

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**TABLE 1**

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<th>Age</th>
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<sup>a</sup>Genotype refers to the genotype of the X chromosome of the F<sub>1</sub> male being tested.

<sup>b</sup>Ambient temperature at which the F<sub>1</sub> male adults are living, either 25°C or 18°C.

<sup>c</sup>Age (in days) is defined as number of days after eclosion, the day of eclosion is Day 0.

<sup>d</sup>Maximum life span refers to the maximum life span in days since eclosion of the F<sub>1</sub> male adult for the genotype and ambient temperature noted.

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A B

![Graph A](image)

![Graph B](image)

**FIGURE 7.**—Amount of antennal $\beta$-gal expression from the 2216 line grown at 25° (A) and 18° (B). Age (in days) is from day 45 from the time of the adults' emergence from the pupal case until near the end of the animals maximal life span. At least 20 different antennae were sampled for each point. Error bars, SEM. To illustrate the periodicity of the changes in levels of $\beta$-gal expression, a line has been drawn connecting the points using a cubic spline function.

the more diffuse type I pattern. Periodic oscillations in the pattern of $\beta$-gal expression, more pronounced at 18° than 25°, can be seen in the quantitative plots shown in Figures 2 and 4. Although more difficult to assess, the period of oscillation also shows a temperature dependence; they are more rapid at 25° than at 18°.

**DISCUSSION**

Two of the main features that characterize the process of aging are the stereotypic predictable manner in which changes occur over time and the timing or rate of progression at which these changes proceed. The documentation of stereotypic predictable patterns in the expression of a number of different genes in the adult *D. melanogaster* provides both a sensitive phenotype for use in examining the aging process as well as an avenue, through the use of molecular genetic techniques, to explore the mechanisms underlying the process of aging (Helfand et al. 1995; Rogina and Helfand 1995).

Observations on the effect on life span of ambient temperature (MiQUEL et al. 1976), genetic mutations that alter the physical activity and oxygen consumption in *D. melanogaster* (TROUT and KAPLAN 1970), physical restriction of houseflies (SOHAL and BUCHAN 1981), and oxidation (ORR and SOHAL 1994; DUDAS and ARKING 1995; SOHAL et al. 1995) all suggest that there is a close relationship between metabolic rate, oxidative state, and longevity (for review see SOHAL and ALLEN 1990). The recent finding of genes and gene products whose temporal patterns of expression scale with respect to life span indicate that the regulation of gene expression may also be coupled to the metabolic rate and/or oxidative state of the adult *D. melanogaster* (Rogina and Helfand 1995; WHEELER et al. 1995). In these studies compensatory changes in the temporal pattern of gene expression or their protein products were seen when life span was altered using ambient temperature, mutations that effect metabolic rate, or mutations that effect the oxidative stress on the animal.

Temperature-shift experiments in *D. subobscura* provided the first evidence that life span may not be directly following ambient temperature/metabolic rate. In these studies it was reported that longevity and the rate of aging over a large portion of the flies' initial life span was independent of temperature (SMITH 1958; CLARKE and SMITH 1961a,b). Subsequently, a number of different investigators have repeated these temperature-shift experiments to determine if there is a temperature-independent phase of aging in *D. melanogaster*; conflicting results are reviewed in MIQUEL et al. (1976). Studies on the temporal pattern of expression of a number of different gene products has also shown a dissociation between the timing of expression of some gene products and life span when life span was altered by ambient temperature or genetic strain differences in *D. melanogaster* (GANETSKY and FLANAGAN 1978).

The characteristic temporal patterns of gene expression seen in *D. melanogaster* provide an opportunity to further examine the relationship between gene expression and the physiological state of the organism. The temporal pattern of expression of those enhancer trap and reporter genes that have been examined in detail have all shown a compensatory alteration in the timing of their expression in response to changes in ambient temperature or metabolic rate (Helfand et al. 1995; ROGINA and HELFAND 1995). This report provides evidence of a gene in *D. melanogaster* whose temporal pattern of expression is independent of ambient temperature and metabolic rate.

The type I pattern of expression seen in the 2216...
enhancer trap line shows a temporal pattern of expression that appears to be independent of ambient temperature and the effects of hyperactivity mutations that increase metabolic rate (Hk' and Sh'). Despite an 11 degree difference in ambient temperature, from 18°C to 29°C, associated with a threefold difference in life span (Figure 3), the time of cessation of type I pattern of 2216's expression is consistently found to be between 40–50 days (Figures 2–5). Increasing the metabolic rate of the animal using Hk' or Sh' mutations confirms that the regulation of expression of 2216's type I pattern is independent of metabolic rate. When the metabolic rate of the animal is increased using Hk' or Sh', the timing of the expression of the type I pattern of 2216's expression is similar to the pattern seen with the animal in a wild-type background (Canton-S, Oregon-R). The type I pattern of expression of 2216 appears to be more closely linked to calendar time/chronological age than to physiological age. Preliminary data have also been obtained on two other enhancer trap marked genes in D. melanogaster whose temporal pattern of expression during adult life appears to be independent of ambient temperature (B. Rogina and S. L. Helfand, unpublished results). Studies to examine the effects of oxidative stress on these reporter genes are presently underway.

The type II pattern of expression of the 2216 gene represents a distinctly different spatial and temporal pattern from the type I pattern. Instead of a broad spatial distribution of β-gal expression throughout the third segment of the antenna, there is focal expression of β-gal in a group of 20–30 cells. In the original 2216 line this pattern of expression is most clearly seen after day 40 (Figure 1B). Animals possessing only one 2216 enhancer trap-containing chromosome have a more marked decline in the level of type I pattern of expression. In these circumstances the type II pattern can be visualized as early as day 5 of adult life. It appears that the type I pattern masks the ability to observe the type II pattern that appears to be present throughout adult life.

The temporal pattern for the type II pattern of expression is also different from type I. Rather than a decline, the level of expression of β-gal in these 20–30 cells changes in a characteristic periodic manner whose period appears to be dependent upon the ambient temperature at which the animal is living. The level of expression in all of the cells appears to change from very intense to completely undetectable. This is best observed after the type I pattern is no longer seen. For example, after day 40 the period of change is ~3–5 days at 25°C and 12–15 days at 18°C. The fact that the periodicity of expression of these cells is longer at 18°C than 25°C suggests that the regulation of expression of the type II pattern of expression may be linked to physiological age. The subtle periodic oscillations in the level of β-gal seen superimposed upon the pattern of decline over the first 40 days of life most likely represents the presence of the type II pattern of expression throughout most of adult life (Figure 2 and 4).

These two distinctly different patterns of expression, type I and II, suggest the existence of differential enhancer elements that regulate both the spatial and the temporal patterns of gene expression in adult D. melanogaster. Evidence for tissue- and cell-specific enhancer elements is abundant during early D. melanogaster development (e.g., cut, Deformed, Ultrabithorax, Antennapedia) (Boulet and Scott 1988; Irvine et al. 1991; Zeng et al. 1994; Jack and Delotto 1995) and age-dependent enhancer elements have been previously described in mammalian systems (Supakar et al. 1993). A final caveat is that these two different patterns of spatial and temporal appearance may be due to regulatory regions from two different genetic loci that are both able to drive the expression of the lacZ reporter from the single enhancer trap insert. This possibility will be evaluated by molecular characterization of the 2216 gene.

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