

Transgenes in the Analysis of Life Span and Fitness

Marc Tatar*

Department of Ecology and Evolutionary Biology, P.O. Box G-W,
Brown University, Providence, Rhode Island 02912

ABSTRACT: *Drosophila P* element-mediated transformation can be used to determine whether and how a specific gene contributes to demographic components of fitness. Motivated by the problem of senescence, researchers have applied this approach to genes thought to affect survival through processes of somatic maintenance. Cu/Zn-superoxide dismutase and catalase reduce the flux of reactive oxygen molecules that are thought to be a central cause of aging. EF1 α is a component of the protein synthesis machine; deterioration of this housekeeping function is a potential contributor to senescence. Molecular chaperones such as the heat shock protein hsp70 are multifunctional molecules that affect a cell's response to acute stress. In some models, senescence results from the cumulative effects of stress, and heat shock proteins may regulate the progress of this deterioration. Transformations with the candidate genes of these proteins were used in independent studies to measure the effect of overexpression on longevity; positive results were reported. Here, I discuss the robustness of these results. I use the studies of superoxide dismutase, catalase, and EF1 α to illustrate how the mutagenic effects of inserts confound our interpretations. I present new data from a reported study of hsp70 overexpression to show how engineered constructs can be used to overcome mutagenic artifacts through the controlled excision of sequences or alleles. The data for hsp70 provide the first strong molecular evidence that somatic maintenance affects longevity. Finally, future potential uses of transformation with *Drosophila* are discussed. I consider how metabolic control theory predicts that overexpression of genes for enzymes of intermediary metabolism is not likely to produce analytically useful changes in components of fitness.

Keywords: senescence, hsp70, superoxide dismutase, *Drosophila melanogaster*, transgenes.

Senescence is a paradox in the study of adaptation. The demographic components of fitness, age-specific survival and reproduction, deteriorate with advancing adult age. This paradox is resolved, at one level, by the fact that natural selection operates with decreased strength at ad-

vanced adult ages (Hamilton 1966; Charlesworth 1994). The decline in age-specific intensity of natural selection permits genes that have deleterious late-age effects to increase in frequency. The early-age effects of these genes may be neutral, in which case senescence evolves through drift, or they may be beneficial, in which case senescence evolves via directional selection. At a proximal level, however, little is known about the identity of such genes or about the mechanisms underlying their evolution. Biometrical studies have documented genetic variance and covariance for age-specific mortality (Hughes and Charlesworth 1994; Promislow et al. 1996; Tatar et al. 1996; Pletcher et al. 1998), but these descriptions may never provide a way to distinguish unambiguously among the ways senescence evolves (Promislow and Tatar 1998; but see Charlesworth and Hughes 1996). This is in part because we first need to resolve which physiological traits lead to the decline in demographic parameters. In what way do these underlying traits function as adaptations at younger ages, whereas at older ages they fail or their negative side effects become overwhelming? To what extent is physiological decline a function of systematic failures that have no apparent trade-off with earlier performance?

The model of the disposable soma provides a specific conceptual framework to address these problems (Kirkwood 1977; Kirkwood and Rose 1991). This model suggests that somatic maintenance regulates life span and senescence. As such, somatic maintenance is an underlying adaptive trait of life histories. The model postulates that organisms inevitably experience and accumulate somatic damage and that the repair and maintenance of the otherwise vulnerable soma is a trait that natural selection can maximize with respect to fitness. Furthermore, it is argued that somatic maintenance is not selected to function beyond some level because increased durability comes at a high marginal cost, most likely to reproduction. This reasoning provides the basis for adaptive tests of senescence: somatic maintenance evolves as a trait to solve the problem of cumulative stress and damage. The evolution of somatic maintenance is constrained by trade-offs, and this constraint results in senescence, an apparently nonadaptive trait.

To evaluate somatic maintenance as an adaptive trait

* E-mail: Marc_Tatar@Brown.edu.

requires two stages of discovery. First, to produce testable hypotheses we must identify specific phenotypes of somatic maintenance and demonstrate experimentally that their failure causes an age-dependent loss in somatic function. (This strategy limits us to evaluating specific candidate maintenance processes rather than the concept of the disposable soma as a whole.) Candidates may involve macromolecule proofreading (Hopfield 1974), reactivation of abnormal proteins by molecular chaperones (Parsell and Lindquist 1994), regulation of nonenzymatic glycation of collagen (Reiser 1990), DNA repair (Bernstein and Bernstein 1991; Promislow 1994), control of free radical formation (Harman 1981; Sohal and Weindruch 1996), macromolecule turnover (Makrides 1983), and the like. Second, we must demonstrate that the expression of the maintenance system is maximized such that further increases in performance would be associated with a net loss in fitness mediated through trade-offs. It is clear, however, that this level of inquiry must first await demonstration that the candidate has the potential to affect somatic maintenance as measured by an increase in survival.

Here I will discuss how transgenic *Drosophila melanogaster* have been used to explore the effect of candidate genes for somatic maintenance on longevity. The pioneering studies involved genes for the proteins Cu/Zn-superoxide dismutase (SOD), catalase, and EF1 α , and they laid the foundation for critical analysis of the effect of gene overexpression on life-history traits. Recent studies have used transgenic strains for the induced form of *hsp70* that yields the 70-kD heat shock protein. Each of these cases will be discussed in this article. I will focus on the criteria necessary to reasonably establish that an introduced transgene improves longevity or age-specific survival, the metric of a well-maintained soma. We shall see that mutations caused by the insertion of the transgene into the host genome causes deleterious effects that are difficult to control and that often compromise the strength of reported conclusions. Given a genetic design with perfect control of mutagenic and other unintended effects, what should we expect from the addition of perhaps a single extra copy of a specific gene, which is perhaps embedded within a metabolic pathway? I will argue that our expectations have been too high. Models of metabolic kinetics suggest that the overexpression of any one gene within a pathway will often have little effect on the net flux of the system. If flux is related to components of fitness, we should expect that increasing the concentration of an enzyme via transgenic manipulation will often have a negligible effect on life span, even if this gene product plays a crucial role in the maintenance of the soma. But as other contributions in this volume demonstrate, many adaptational hypotheses are indeed amenable to transgenic analyses. The nature of

the manipulated genes in these cases can be used to suggest when we can expect the technique to be profitable.

Oxidative Stress Defense Enzymes

In aerobes, free radicals are produced at a rate of 2%–3% of the oxygen consumed by cells, and molecules such as superoxide and hydrogen peroxide further react to produce widespread molecular and cellular damage (Stadtman 1992). The antioxidant enzymes SOD and catalase permit cells to function despite this steady flux of reactive oxygen metabolites. It is thought, however, that these enzymatic defenses function at a level that is less than their maximum capacity such that somatic damage accumulates and eventually accelerates senescence (Sohal and Weindruch 1996). In the context of the disposable soma, these and other oxidative defenses could be improved by natural selection, but only with a cost exacted through trade-offs with reproduction, development, or current performance. This reasoning presents a testable adaptive hypothesis: overexpression of antioxidative enzymes will increase life span, but at the same time it will reduce other elements of fitness, such as the rates of reproduction or development.

The first aspect of this argument, that expression of antioxidant genes can affect survival, has been studied both with mutant and transgenic strains of *Drosophila melanogaster*. Mutants of *SOD* and *catalase* with enzyme activity reduced to as low as 50% of wild type are phenotypically normal with respect to oxidative defense and adult survivorship (Mackay and Bewley 1989; Phillips et al. 1989; Seto et al. 1990; Staveley et al. 1990; Orr and Sohal 1992, 1993; Orr et al. 1992). Null mutants have greatly impaired defense and reduced longevity (Phillips et al. 1989). To study the effect of overexpression, transgenic strains were constructed to contain single additional copies of either *SOD* or *catalase* (Seto et al. 1990; Orr and Sohal 1992, 1993). Transformed strains always increased whole-animal levels of enzyme activity but failed to improve longevity. The proteins SOD and catalase, however, act sequentially to remove superoxide anion radicals to H₂O₂, and then H₂O₂ to water and molecular oxygen. Since the tandem overexpression of these genes may be required to elicit improved somatic maintenance, Orr and Sohal (1994) subsequently evaluated transgenic lines containing single additional copies of both *SOD* and *catalase*. These double-transforming strains were reported to increase life span by 34%, reduce protein oxidative damage, and enhance late-life physical performance. The fundamental notion that oxidative defense affects life span finally appeared to receive unequivocal support.

The matter, however, was not resolved. Rather, a fundamental problem for the transgenic analysis of life histories came to light when the design of Orr and Sohal

(1994) was considered in detail (Tower 1996). Orr and Sohal constructed 15 *SOD/catalase* transgenic lines, each within a nearly isogenic background. The mean life spans of these lines ranged from 47 to 71 d (fig. 1A). On the basis of these measures, Orr and Sohal chose the three longest-lived lines for statistical comparison of survival and performance relative to a single control line (Orr and Sohal 1994, p. 1129). The control line had double *P* element inserts containing the eye-color marker *ry*⁺ but lacking transgenic *SOD* and *catalase*. This selective use of data was reasoned on the basis that each of the excluded lines may not have fully expressed the transgene owing to *cis*-regulatory position effects (see Tower 1996 for a discussion of this assumption). Unfortunately, the specific expression of the transgenes were not measured at any point.

A possible explanation for the pattern reported by Orr and Sohal (1994) follows from the observation that insertion of *P* elements often suppresses longevity (Clark and Guadalupe 1995; discussed more fully later). Since Orr and Sohal considered only a single representative of the control genotype, this line most likely possessed a reduced-longevity phenotype. Among the more numerous treatment strains, the mutagenic effect of inserts is expected to have decreased the sample mean of longevity. But some lines in the sample are expected to remain relatively robust because their inserts fell into transcriptionally inactive positions, and such lines are likely to have represented the three selected by Orr and Sohal. This explanation suggests a general way to minimize the confounding effect of insert mutagenesis: randomly replicate the control strains and then compare the mean longevity of this sample to that of a full sample of treatment strains. The data of Orr and Sohal (kindly provided by W. R. Orr)

permit us to estimate the number of replicate strains necessary to conduct such a test. Based on the observed among-line variance seen in figure 1A, we need at least 15 strains of both the control and treatment genotypes to detect a 10% difference in genotype longevity when the power of the test is 0.90 and $\alpha = 0.05$ (Sokal and Rohlf 1981).

Elongation Factor

Insertional mutagenesis also emerges as a central issue in the extensive studies of the elongation factor protein (*EF1 α*) as a factor involved in somatic maintenance (Shepherd et al. 1989; Stearns and Kaiser 1993, 1996; Stearns et al. 1993; Shikama et al. 1994; Kaiser et al. 1997). *EF1 α* catalyses tRNA binding to ribosomes. It is a typical house-keeping molecule contributing to the cell maintenance and protein turnover. In *Drosophila melanogaster*, the synthesis of the *EF1 α* protein sharply declines with age, evidently because of a decrease in *EF1 α* transcription (Webster and Webster 1984; Webster 1988). These changes precede by several days an age-related decline in total protein synthesis (Webster and Webster 1983). These facts suggest *EF1 α* as a candidate gene that may contribute to the duration of life span.

Shepherd et al. (1989) evaluated the effect of *EF1 α* on *D. melanogaster* adult longevity by constructing flies that possessed an additional copy of *EF1 α* under the control of a heat-inducible *hsp70* promoter. Constructs with promoter-*EF1 α* -*ry*⁺ were introduced by *P* element-mediated germ-line transfection of *rosy-506* flies. A single treatment line was compared to a single control line. The control contained a selectable *P* element construct containing only

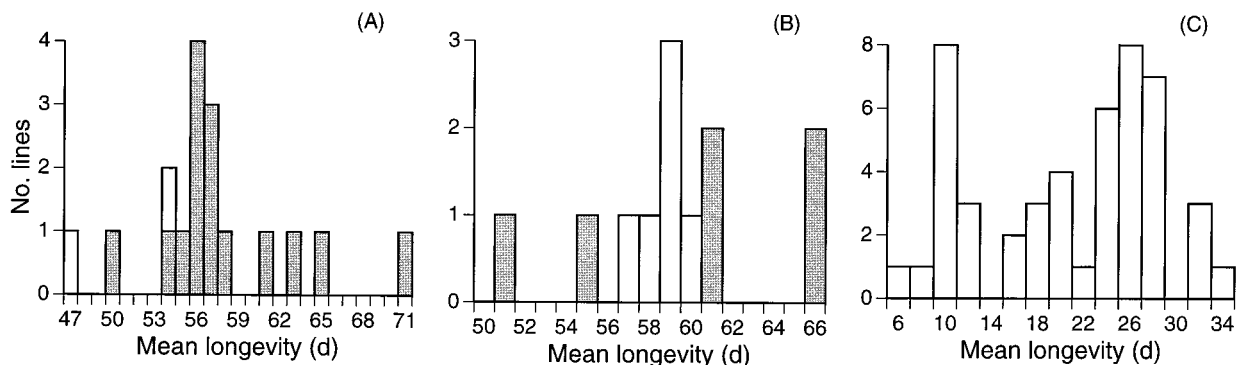


Figure 1: Longevity for strains mutagenized by *P* element constructs with and without transgenes. *A*, Males of strains with double transgenic inserts from Orr and Sohal (1994). *Shaded*, *SOD/catalase* constructs; *open*, control constructs (unpublished data of results reported in Orr and Sohal 1994). *B*, Males of strains with single transgenic inserts from Kaiser et al. (1997) at 25.5°C. *Shaded*, *Ef1 α* constructs (11.8 kb); *open*, control constructs (9.9 kb; unpublished data of results reported in Kaiser et al. 1997). *C*, Males of strains with simple *P* element inserts measured at 25°C (from Clark and Guadalupe 1995).

ry⁺; the insert was at a site independent of the site in the treatment strain. Flies were reared both at 25°C, a nonexpressive temperature, and at 29.5°C, where Shepherd et al. expected the *hsp70* promoter to initiate transcription of the transgenic *EF1α*. The strategy was to compare the treatment genotype to itself across conditions where *EF1α* would or would not be expressed. By measuring longevity differences within a single genotype, the effect of transgenic expression might be separated from the insert mutagenic effects, which varies only among genotypes. The control strain served to scale the effect of temperature on life span independent of *EF1α* expression. When the results are plotted as in figure 2A, we can recognize this design as an assay for gene-by-environment interaction. The higher temperature reduced longevity in both genotypes, and the treatment strain exhibited superior longevity relative to control strain. Critical to the hypothesis, the proportional difference between genotypes was greater at 29.5°C, and from this observation Shepherd et al. argued that *EF1α* expression increased longevity.

These results were encouraging, but we can now recognize that the strategy did not overcome the problem of insertional mutagenesis. (I put aside the issue of comparing proportional vs. absolute differences.) The data of Shepherd et al. (1989) cannot distinguish between an effect due to heat-induced expression of the transgenic *EF1α* gene and one due to temperature sensitivity of genes that were mutated when *P* elements were inserted onto the host chromosomes. Insertional mutagenic effects are evident from the data at 25°C, where both the control and treatment strains only express the *trans*-marker (*ry*⁺), yet the longevity of these genotypes differed by 3 d. Evidently

the insert present in the control strain was deleterious relative to that of the treatment strain. Recall that the control and treatment inserts varied both in location and size. Further, if the deleterious effect of the mutation carried by the control flies was temperature sensitive, we could not distinguish whether longevity was reduced in the control strain at 29°C or improved in the treatment strain when *EF1α* was supposed to be overexpressed.

The essence of this issue was subsequently addressed in the laboratories of S. Stearns and C. Brack. These groups aimed to study the pleiotropic relationship between *EF1α*-promoted longevity and other life-history traits (Stearns and Kaiser 1993, 1996; Stearns et al. 1993; Kaiser et al. 1997). Aware of confounding effects of insert mutagenesis, Stearns and colleagues worked from the original strains of Shepherd et al. (1989) to replicate the control and treatment genotypes. Various inserts were generated at unique third chromosome sites, all within a common inbred background. Life tables were compiled for each strain at 25°C and 29.5°C. Contrary to the conclusions of Shepherd et al., the data revealed no significant main or genotype-by-temperature interaction effects (fig. 2B; ANOVA, $P > .07$ in all cases; unpublished data provided by S. C. Stearns and M. Kaiser). Contemporaneously, Brack and colleagues (Shikama et al. 1994) measured transgene transcription in the lines of Shepherd et al. The transgenic construct was not expressed at 29.5°C, which could have been anticipated since the *hsp70* promoter typically is not induced at this temperature. The replicate strains of Stearns subsequently showed the same lack of expression (Kaiser et al. 1997). It is apparent, therefore, that these strains represent a series of *P* element inserts that varied in construct length (9.9

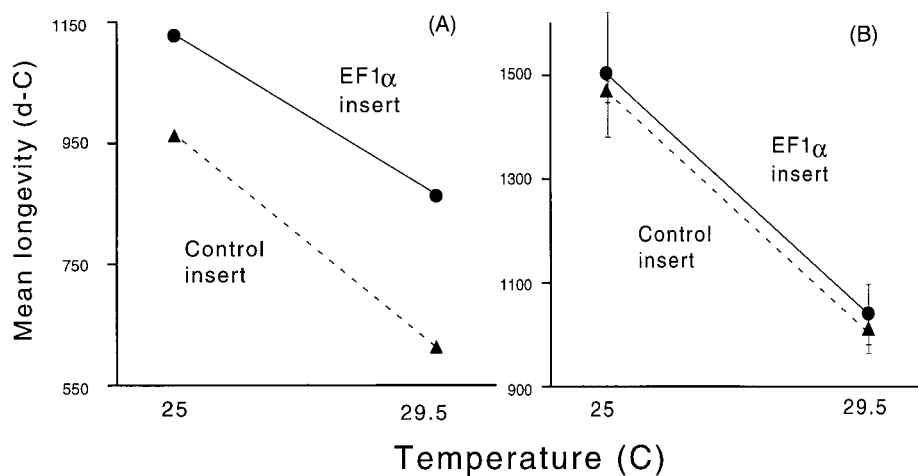


Figure 2: Interaction of transgene strain with rearing temperature in two studies of male longevity. Strains possessed inserts with and without *Ef1α*. Longevity is plotted as day-degrees to provide a common scale across temperature treatments. Sources: A, Shepherd et al. 1989; B, data of “position experiments” from Kaiser et al. 1997 (S. C. Stearns and M. Kaiser, unpublished data).

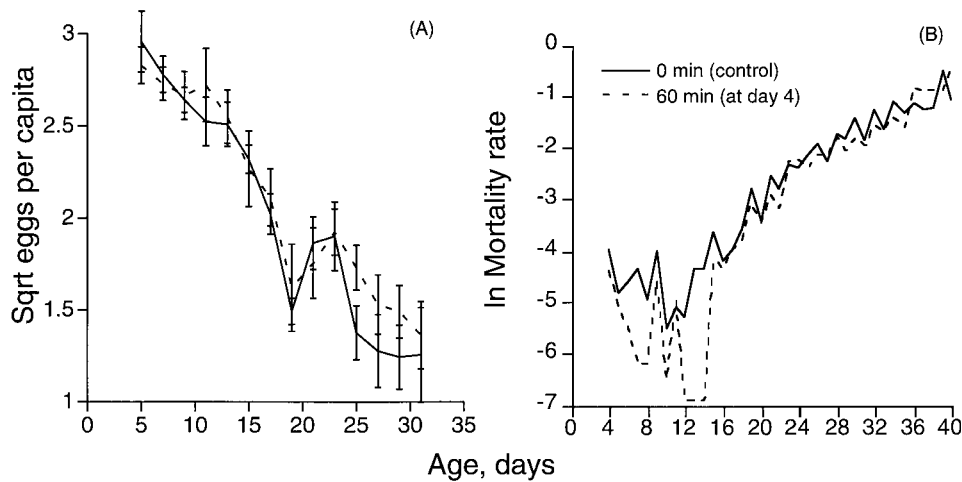


Figure 3: Demographic consequences of a brief heat shock on female *Drosophila melanogaster*. In each cohort, 900 4-d-old females of an inbred strain (JWC24) were either heated for 60 min at 36°C or retained at the control temperature of 24°C. Subsequent mortality and fecundity rates were measured at 24°C. A, Eggs laid per female conditional on survival; B, logarithm of mortality rate estimated as $\ln(-\ln[p_x])$, where p_x is the age-specific probability of survival (A. A. Khazaeli and M. Tatar, unpublished data).

kb in control strain, 11.9 kb in treatment strain) and position of integration. Their effects on longevity are largely random and can best be understood in terms of their mutagenic action (Kaiser et al. 1997).

The consequences of *P* element mutation longevity is not surprising in light of Mackay's extensive work showing the effects of *P* elements on larval viability and adult morphology (Mackay 1989; Mackay et al. 1992). However, the consequences of *P* elements on adult life history was not appreciated until Clark and Guadalupe (1995) investigated how simple inserts affected longevity. They generated 48 *P* element-tagged strains with a construct containing the *white* minigene. Because of the rescue of eye color in the transformed flies, the host strain could not serve as an adequate control from which to estimate mean mutagenic effects on longevity. However, the distribution of longevity among the mutant lines is instructive (fig. 1C). There was substantial variation among the lines, and many of the lines were short-lived relative to what we expect for laboratory-cultured flies. These features reinforce our cautious interpretations of the *SOD*, *catalase*, and *EF1 α* transgenics: insertional mutagenesis is a strong force that can generate large differences among treatment and control lines, independent of any differential expression of transgenes.

Heat Shock Proteins

In some of the earliest experimental studies of life history, Maynard Smith (1958) found that life expectancy was dramatically improved in female *Drosophila subobscura* when

they were first maintained at elevated temperature for 5–12 d. This effect was associated with atrophied reproductive tissue, attributed to the prolonged heating of the females. In contrast, recent experiments with *Drosophila melanogaster* (Khazaeli et al. 1997) and with the nematode *Ceanorhabditis elegans* (Lithgow et al. 1995) revealed that life expectancy is increased by very brief, acute heat shocks (e.g., 15 min at 37°C). In a related discovery, long-lived mutant strains of *C. elegans* were found to possess exceptional intrinsic tolerance to acute stress in the form of heat as well as UV radiation (Lithgow et al. 1994, 1995; Murakami and Johnson 1996). Since thermal stress is a primary inducer of the so-called heat shock proteins (see Parsell and Lindquist 1994; Feder 1999, in this issue), these observations suggest a means to evaluate an intriguing but as yet untested hypothesis: heat shock proteins in their capacity as molecular chaperones regulate the durability of the soma in the face of chronic internal and external stress (Jazwinski 1996; Lithgow and Kirkwood 1996).

Before testing whether heat shock proteins affect longevity, it was recognized that heat-induced longevity might simply result from reduced mortality costs of egg production. Khazaeli and colleagues (A. A. Khazaeli and M. Tatar, unpublished data) addressed this possibility in a series of studies with *D. melanogaster*. Female flies were briefly heated at age 4 d, and their schedules of egg production and age-specific mortality were compared with those for unheated flies. Longevity was extended in the heat-treated flies in the typical manner: age-specific mortality is reduced by a factor of two over a period of 14–21 d following the heat treatment (fig. 3A). Contrary to the

mortality cost hypothesis, egg production did not differ among the cohorts (fig. 3). However, the brief heat shocks did reduce fertility (fig. 4). Females paired with unheated males began to lay eggs of reduced viability 4–6 d after heat shock. Male fertility was reduced immediately after exposure to heat. Thus, there are both positive and negative fitness consequences of exposure to heat, but we cannot simply explain heat-extended longevity to be a result of reduced egg number. Also, although heat treatment affects egg quality, it is generally unknown whether variation in egg size or content can generate differences in female mortality. It is prudent then to consider the alternative hypothesis that heat shock proteins play a direct role in the extension of longevity.

Heat shock or “stress” proteins are a conserved class of molecules with a broad range of chaperone functions that include preventing the aggregation of denatured proteins, the promotion of protein folding, and protein transport and degradation (Morimoto et al. 1994; Parsell and Lind-

quist 1994). When under inducible regulatory expression they play important roles in the response to both internal and external stress caused by factors such as heat (Feder 1999, in this issue), free radicals (Wheeler et al. 1995), inflammation (Ezzell 1995), and molecular toxins (Parsell and Lindquist 1994; Feige et al. 1996). Because of their potential to protect the soma under specific stresses, it is widely thought that heat shock proteins contribute to the everyday persistence of the soma as reflected in the organismal rate of senescence (Heydari et al. 1994; Jazwinski 1996; Lithgow and Kirkwood 1996). However, studies of association between hsp expression and age provide ambiguous support for this hypothesis. In rats, levels of the 70-kD heat shock protein (hsp70) are reduced by 50% in hepatocytes freshly isolated from old relative to young animals (Heydari et al. 1994), likely as a result of altered binding activity of heat shock transcription factor (Heydari et al. 1996). *Drosophila melanogaster* presents a contrasting pattern of age-dependent change (Wheeler et al. 1995);

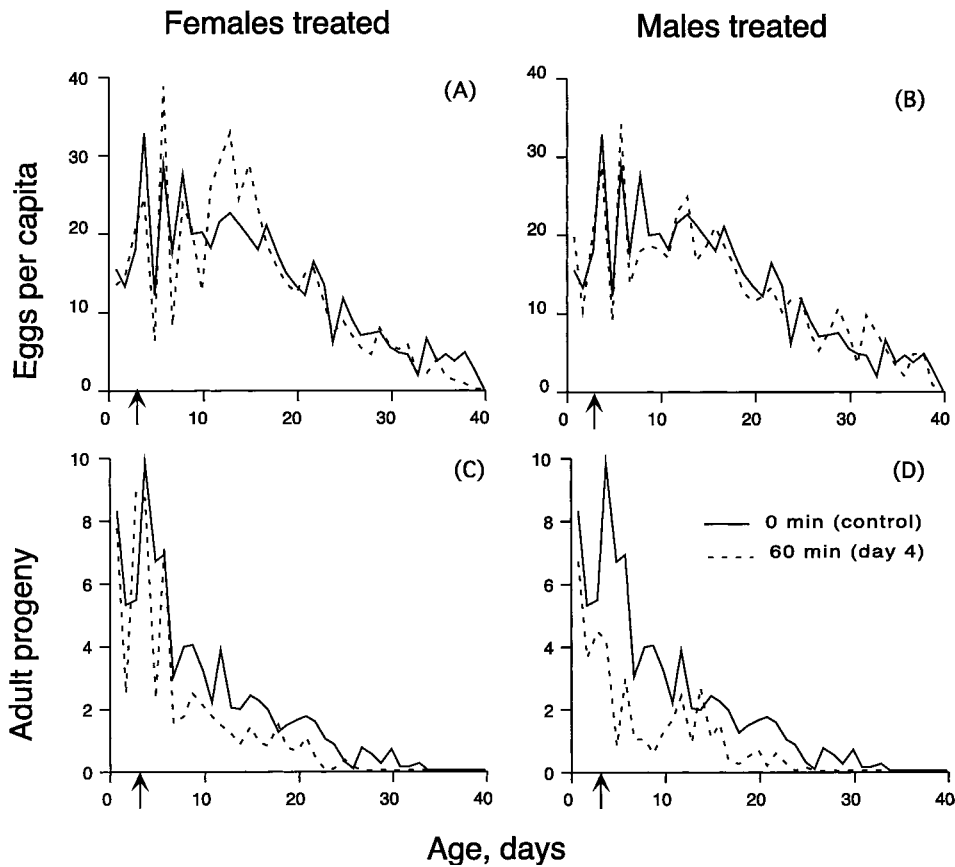


Figure 4: Effect of brief heat shocks on progeny. Virgin 4-d-old adults were either heated for 60 min at 36°C or retained at 24°C. Subsequently at 24°C, individuals of each sex were paired into shell vials with an unheated mate (A, C, treated females; B, D, treated males). Pairs were transferred to fresh vials daily. The laid eggs were counted (A, B), and the vials were retained to score the number of progeny (C, D) (A. A. Khazaeli and M. Tatar, unpublished data).

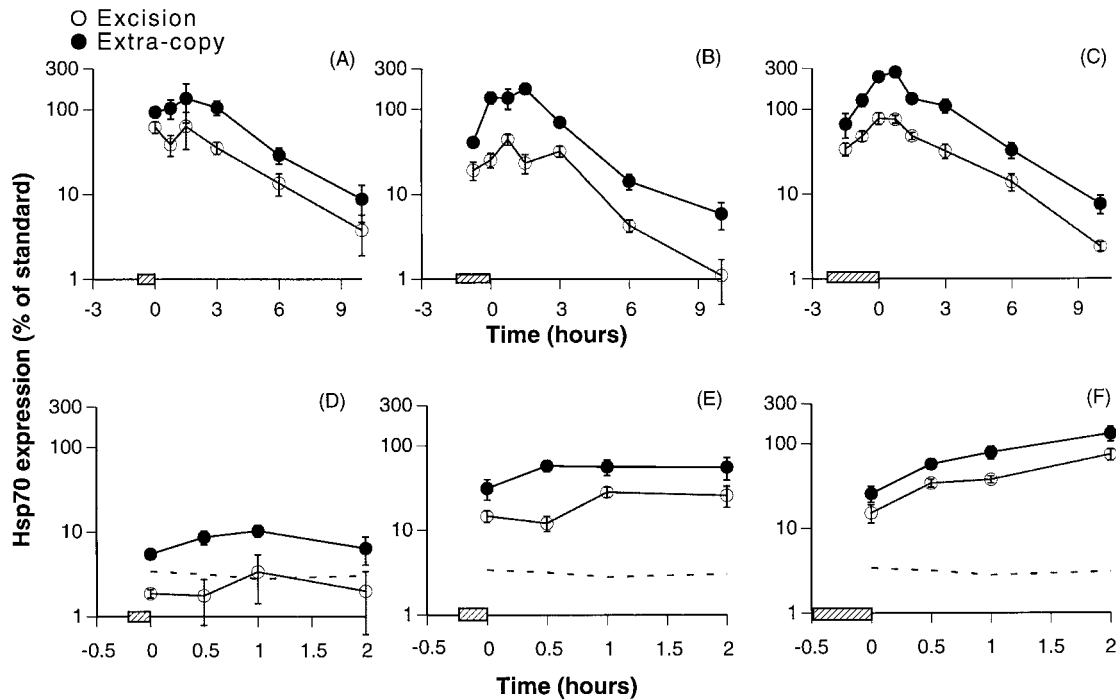


Figure 5: Expression kinetics of Hsp70 protein in 4-d-old male flies upon heat treatment (36°C) measured by ELISA with primary antibody 7FB. Expression is relative to an external standard lysate made from heat-shocked embryos. Transgenic extra-copy flies carried 12 additional copies of the inducible *hsp70* gene. Excision flies carried a remnant insert at the same integration site. Each point is derived from three or more assayed flies (SE on this mean). Bar on abscissa shows the period of heat treatment: A–C, 45, 90, and 135 min, respectively; D–F, 10, 20, and 30 min, respectively. Dashed line indicates the mean expression of unheated flies, genotypes combined (unpublished data of results reported in Tatar et al. 1997).

transcription of *hsp22* and *hsp23* increases with age, while *hsp70* accumulates owing to as yet undescribed posttranscriptional changes. These data together suggest an association of hsp70 with aging, but they do not establish whether the chaperones play a causal role.

Transgenic analysis of the heat-induced longevity phenotype of *D. melanogaster* is a powerful way to evaluate the effect of heat shock proteins on senescence, provided care is taken to control for the pervasive effects of insertional mutagenesis. Welte et al. (1993) introduced a molecular technique for such control. Homologous recombination was used to produce allelic strains that shared a common integration site but that differed in copy number of *hsp70* transgenes. Germ lines of the white-eyed strain w^{1118} were transfected with *P* element constructs possessing multiple copies of *hsp70* and a w^{hs} eye color selection marker. These genes were flanked by yeast FRT sequences. Unequal homologous recombination was induced with FLP recombinase, which resulted in one chromosome with a loss of the between-FRT sequences including the *hsp70* (excision chromosome) and the other chromosome with a gain in the between-FRT sequences (extra-copy chromosome). Crosses of recombinant offspring through bal-

ancer stocks generated paired sets of homozygous recombinant strains. The excision strain contained the wild-type complement of inducible *hsp70* (10 copies) and a remnant *P* element insert. The extra-copy strain contained the *hsp70* wild-type complement plus 12 copies of *hsp70* at the integration site common to the pair. Welte et al. (1993) and Feder et al. (1997) used these flies to demonstrate unambiguously that *hsp70* expression mediates thermotolerance in embryos, larvae, and pupae.

The effect of *hsp70* on senescence has also been investigated (Tatar et al. 1997). In a series of trials, 4-d-old males of excision and extra-copy flies (a chromosome 2 integration site strain) were exposed to 36°C for durations of 10–135 min. (for general methodology, see Khazaeli et al. 1997). The quantity of *hsp70* expressed following heat shock (fig. 5) was measured immunologically by ELISA with a primary antibody specific to heat-inducible *hsp70* (see Feder et al. 1997 for discussion of ELISA methods). Percentage expression was measured relative to a standard control lysate made from heat-stressed embryos. In all cases *hsp70* expression was greater in extra-copy flies, and the concentration of induced protein returned to background levels within 12 h of heat shock. Proportional haz-

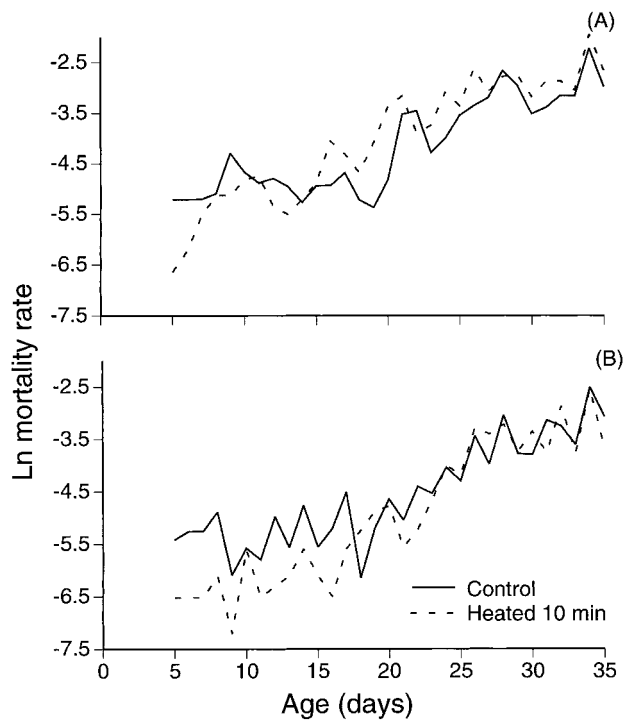


Figure 6: Mortality rates of *hsp70* transgenic males at 24°C for 30 d following a 10-min heat shock at 36°C. A, Excision strain with wild-type copy number of *hsp70*; B, extra-copy strain with 12 additional copies of *hsp70*. Logarithm of mortality is estimated as $\ln(-\ln[p_x])$, 1,100–1,400 males per cohort (unpublished data of results reported in Tatar et al. 1997).

ard analysis was used to quantify within strains the extent to which heat treatment improved the age-specific survival of heated versus unheated flies. The proportional hazard regression estimates the ratio differences among mortality rates attributable to continuous or discrete covariates (Lee 1992). Age-specific survival was analyzed over the 2 wk following heat shock treatment as this is the typical duration for the effect of a single heat treatment (Khazaeli et al. 1997). By comparing the magnitude of the proportional hazard ratios among strains, we evaluated whether *hsp70* copy number increased longevity following heat shock.

The most revealing trials were those for the shortest heat durations. For treatments of 10 or 15 min, only the extra-copy flies expressed inducible *hsp70* (fig. 5D), and only these flies demonstrated reduced mortality rates (fig. 6). Since extra-copy and excision flies experienced the same thermal treatments, these trials demonstrate that the expression of *hsp70* itself is sufficient to extend longevity, presumably against a background of other heat-inducible responses. Furthermore, it appears that *hsp70* had an indirect effect on somatic persistence since the effect of *hsp70*

on mortality was persistent relative to the very transient nature of the protein, based on whole-animal assays. In contrast to these brief thermal treatments, longer heat periods produced similar levels of heat induced longevity in excision and extra-copy flies (fig. 7), which coincided with substantial levels of induced *hsp70* expression in both strains (fig. 5). Finally, the longest heat exposure, that of 135 minutes, induced the highest observed levels of *hsp70* (fig. 5C) but did not improve age-specific survival (fig. 7).

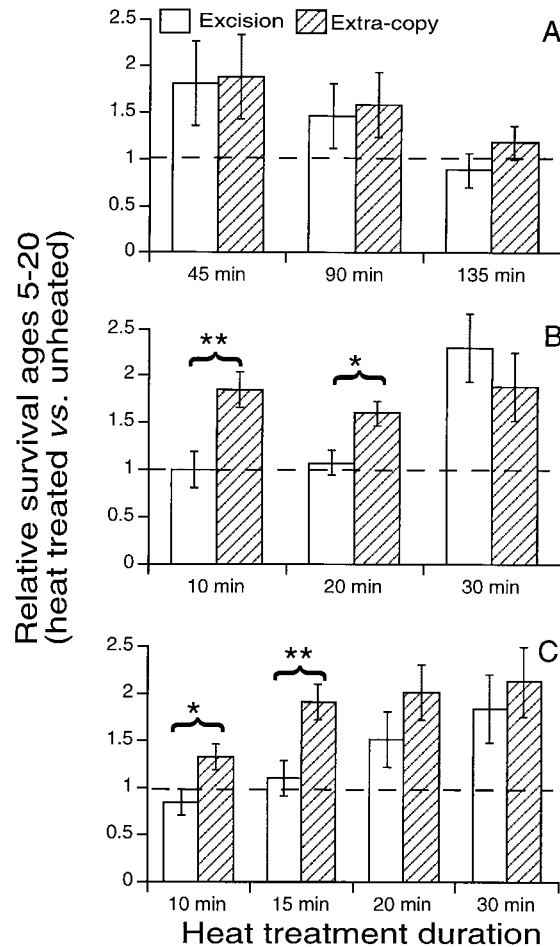


Figure 7: Relative survival upon heat shock of extra-copy and excision strains. In all trials, relative survival was estimated with proportional-hazard regression as the proportional increase in age-specific survival over ages 5–20 s in the heat-treated cohort relative to the unheated cohort of the same strain (with SE). A ratio of 1.0 indicates that survival is similar among the heated and unheated flies. Values significantly >1.0 (likelihood-ratio test, $P < .05$) occurred in all cases, except for flies of both strains heated 135 min. A, Excision flies heated 10 and 20 min; B, excision flies heated 10 and 15 min; C, brackets indicate significant differences between the excision and extra-copy strains for a given heat treatment duration (factorial proportional-hazard regression; asterisk, $P < .05$; double asterisk, $P < .01$, significance corrected for multiple comparisons). (Unpublished data of results reported in Tatar et al. 1997.)

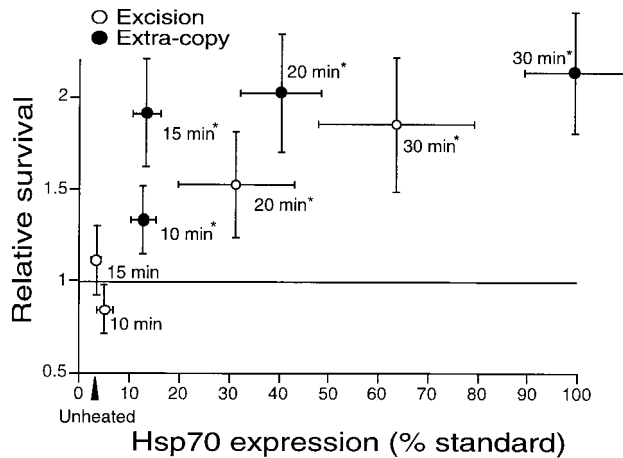


Figure 8: Effect of Hsp70 protein on relative survival in extra-copy and excision strains. Expression of hsp70 measured by ELISA from flies sampled 1 h following the return of heated cohort cages to 24°C and from the unheated control cohorts. Standard errors presented for estimates of both variables. Log(relative survival) regressed on log(hsp70 expression) is positive and linear for both strains (ANCOVA, $F = 8.76$, $df = 1$, $P < .023$) but does not differ significantly among the strains (ANCOVA, strain effect, $F = 2.44$, $df = 1$, $P = .18$). (Reprinted with permission from Tatar et al. 1997.)

The kinetics of hsp70 induction offers a way to attribute extended longevity to *hsp70* transcription even though the strains vary by transgene insert length, selection markers, and genetic background. The strains appear to possess similar dose-response functions describing gene-to-phenotype expression (fig. 8). Therefore, differences among the strains in their induced longevity can be understood in terms of differences in their heat dose sensitivity. Across strains, we found that survival was initially enhanced when hsp70 levels exceeded about 10%–15% of standard. Given a very brief heat treatment, this threshold was surpassed by extra-copy but not by excision flies. However, in both strains, longer heat treatments induced hsp70 expression adequate to extend longevity up to the maximum value of about twofold improvement. While the strains vary in some minor genetic ways, we can predictably explain the longevity extension as the effect of *hsp70* transcription with respect to the effective threshold of the protein.

These data were the first to demonstrate a direct effect of hsp70 upon longevity. As hypothesized, heat shock proteins play a role in the regulation of senescence. The details of this role are presently unknown; they most likely involve many cellular systems that are modified by transient heat shock protein expression. At least we may now conclude that somatic maintenance contributes to the regulation of mortality rates and longevity, essential components of demographic fitness.

Transgenes and Metabolic Control Theory

As the work with hsp70 demonstrates here and in Feder (1999, in this issue), transgenic analyses can be a powerful tool to infer the genetic control of complex traits related to adaptation. The strategy, however, may be limited even when it is executed in a genetically ideal manner. It is likely that overexpression of some broad classes of genes will not appreciably affect fitness phenotypes even though the genes are intimately involved in the traits. In this final section I will discuss how metabolic control theory may predict that constitutively expressed genes involved with intermediary metabolism are not likely to be amenable to analysis by transgenic overexpression.

Kacser and Burns (1973, 1981) showed how a concave function describes a saturation kinetics of enzyme activity on metabolic flux. Many enzymes of intermediary metabolism display this kinetic, with the result that individual enzymes embedded in a multilocus pathway are effectively insensitive to changes in their levels of activity over a broad range (fig. 9). The flux curve is concave and plateaus as a result of the interactions between successive steps of enzymes linked by shared substrate and products. The wild-type level of enzymatic activity is expected to reside well on the plateau of the saturation curve since almost all enzymes are in excess with respect to their contribution to the flux of the system. This model provides a plausible basis for the ubiquitous occurrence of the dominance of wild type over mutant alleles (Kacser and Burns 1981), as well as the apparent near neutrality of much allelic variation (Hartl et al. 1985). In the context of the current discussion, the model also suggests that the overexpression

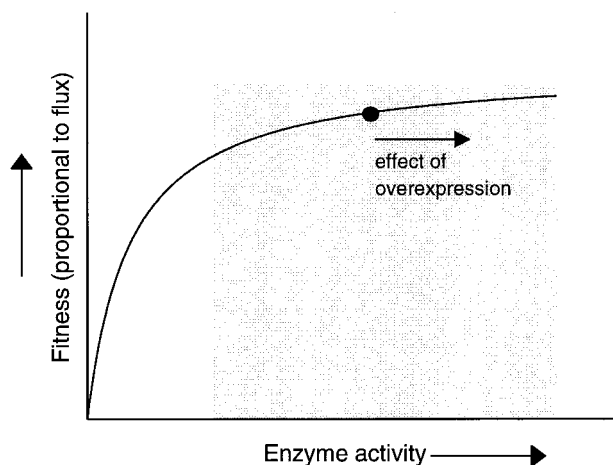


Figure 9: Idealized enzyme saturation function after Kacser and Burns (1981). In the shaded region, variation in individual enzyme activity is nearly undetectable. The point represents the expected value of an average wild-type enzyme activity.

of a single gene may not increase the value of complex phenotypes, especially those, such as cellular housekeeping and somatic maintenance traits, that involve metabolic flux. A gene may indeed contribute to the fundamental expression of a phenotype, and transgenic addition of an allele can lead to increased level of the target protein, but this may not be sufficient to cause a corresponding increase in the phenotype. Overexpression of the gene pushes the protein activity along the plateau of the saturation curve to the right of the wild-type level of activity (fig. 9). Contrary to some expectations, we might even observe a decrease in the trait value if there are substantial metabolic costs of this overexpression.

Models of saturation behavior were originally developed for linear metabolic pathways with fixed initial and terminal pools and without saturation of the reaction (Kacser and Burns 1973, 1981). Can deductions from this theory be extended to nonlinear metabolic networks or to demographic phenotypes related to fitness? In theory, models of pathways that involve feedback inhibition will present some metabolic steps with relatively high sensitivity (Savageau 1976), and under these conditions flux will change proportionally with the activity of this enzyme. In such cases, however, the sensitivity of all other elements of the pathway will be very small since the sum over all steps must equal unity (Kacser and Burns 1973). In particular, continuous traits that are described by multilocus inheritance can be modeled with the majority of intermediary enzymes at plateau-level activities that can be expected to show little response to transgenic overexpression (Kacser and Burns 1981).

The situation can be illustrated by the dosage response *Drosophila melanogaster* genotypes for resistance to exogenous oxidizing agents. In several studies, resistance to paraquat was measured for mutant and transgenic strains that possessed different levels of SOD activity. Combining these data sets, we observe a concave functional relationship between SOD activity and resistance, measured as survival (fig. 10). More than a 40% loss of SOD activity was required to reduce resistance to paraquat. Transgenic overexpression of SOD raised enzyme activity by 30%–40%, but resistance was either unaffected or reduced. Longevity under normal environmental conditions was also measured for each of these strains. Only the complete deficiency mutant *cSOD¹⁰⁸* exhibited reduced life expectancy (Phillips et al. 1989). Single-gene transgenic lines were not observed to improve longevity. Similar saturation kinetics can be described for mutants and transgenics of *catalase* with respect to resistance to hydrogen peroxide and to life expectancy (Mackay and Bewley 1989; Orr et al. 1992).

The conformity of these data to saturation-like kinetic behavior could explain why the overexpression of single

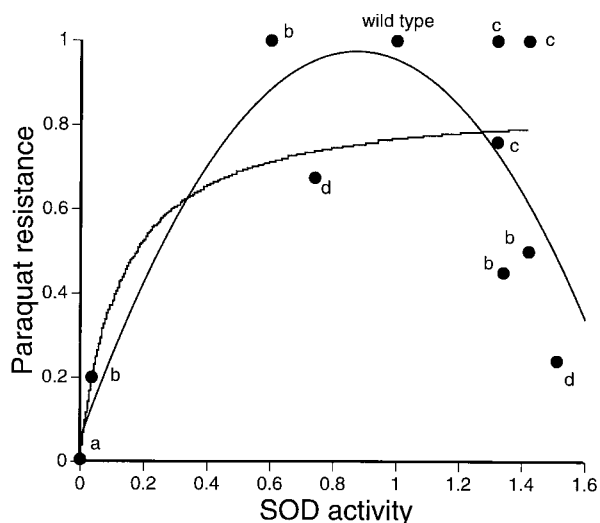


Figure 10: Resistance to paraquat as a function of superoxide dismutase activity. Both variables are standardized relative to the observed value of the wild type used in each of four studies of mutant and transgenic strains. Sources: point *a*, Phillips et al. 1989; points *b*, Seto et al. 1990; points *c*, Orr and Sohal 1993; points *d*, Staveley et al. 1990. The quadratic function was fit with all data; the hyperbolic function was fit to a set exclusive of the values from high active genotypes in Seto et al. 1990 and Staveley et al. 1990.

genes in the oxygen radical conversion pathway have been ineffective. Might the joint overexpression of SOD and catalase overcome this lack of response, as Orr and Sohal (1994) have proposed? Metabolic control theory suggests that the flux of two enzymes embedded in a linear pathway will produce saturation behavior on a two-dimensional plateau (Clark 1991). Potentially the relative shortness of the oxygen radical control pathway may restore a proportional response, but this proposal still awaits rigorous empirical study.

Conclusion

The studies presented in this volume illustrate that transgenic analysis of adaptation can be productive despite the potential caveat of metabolic kinetics. These cases suggest categories of genes and traits that are amenable to the approach.

Inducible Traits

Transgenes may alter the sensitivity of an induced response, with or without affecting the potential maximum level of transcript. As discussed in this volume, the inducible gene *hsp70* of *Drosophila melanogaster* is tightly regulated (Feder et al. 1992) such that little protein is

normally present in cells. Transcription is rapidly initiated upon heat shock. In terms of saturation kinetics, manipulation of this response permits comparisons between an effectively null-activity state and a corresponding high-activity state. The transgenic addition of *hsp70* does not so much change the total expressible level of protein as it changes how much protein could be produced at each heat dose.

Regulatory Genes and Signal Transduction

The expression of many phenotypes depends on the timing and duration of regulated transcription. Both mutational and transgenic analyses are extremely powerful methods of revealing the phenotypic consequences of genes involved with this regulation. Transgenic analysis is efficient here because it experimentally alters the state of a system. This strategy was employed by Schmitt et al. (1995) in their analysis of the shade avoidance response. Phytochrome A in seedlings mediates a far-red/high-irradiance response that inhibits hypocotyl extension. In tobacco plants the constitutive expression of a transgenic oat *phyA* gene counteracts the normal down regulation of the gene during development and blocks the wild-type stem elongation response to far-red signals typical of older plants. Schmitt et al. (1995) used these transgenic plants to measure the fitness consequences of failure to elongate under conditions that would usually stimulate this plastic response.

Tests of Allelism by Transgenic Complementation

Mutagenesis can produce phenotypes useful for the study of adaptation. It is not trivial, however, to accurately ascribe the effect to an allele at the locus of interest rather than to changes in closely linked loci or nontranscribed regions. When it is possible to clone the putative mutant allele, the allele can be transformed into a test genome. Without its original linkage, it is possible to directly assess the effect of the allele as it complements the alleles of the host genotype. Purrington and Bergelson (1999, in this issue) illustrated the approach in their study of the chlorsulfuron-resistance allele of *Arabidopsis thaliana*. The transgenic addition of a resistance-conferring allele, originally isolated from a mutational screen, restored viability in the presence of chlorsulfuron and produced pleiotropic decrements in seed production.

Future Directions

Future applications of transgenic analysis of fitness will expand on these precedents. For instance, in quantitative trait loci (QTL) mapping, tens to hundreds of genes typ-

ically fall within a mapped interval. Candidate genes that are suspected to influence the trait may occur in the region, and we would like to know whether alleles at these loci are responsible for the QTL's effect. It may be possible to directly evaluate the allelic effects of a candidate genes with transgenesis. For instance, Curtsinger and colleagues (Resler et al. 1998) have resolved a QTL on the third chromosome of *D. melanogaster*, which explains about 30% of the selection response between the control and postponed senescence selection lines of Luckinbill et al. (1984). Several longevity assurance candidate genes occur within the confidence interval of this QTL, including *Cu/ZnSOD* and *hsp68*. Are alleles at these candidate loci responsible for the observed selection response? As a result of selection, we can assume that alternative alleles affect senescence in the control versus long-lived strains. Therefore, clones of these alleles from the respective selection lines could provide material to transform a stock that is otherwise deficient at the candidate locus. Rescue of the transformed strains toward the appropriate selection line value would implicate the candidate gene as the actual quantitative trait locus.

The future also promises improved control of transformation-related artifacts. This is especially important for the study of life-history and fitness traits since these are so sensitive to positional and mutational effects of the insert and to genetic background. For instance, Siegal and Hartl (1996) developed a system for the coplacement of alternative alleles at identical sites within a single genetic background. Flies are transformed with a *P* element construct that contains both alleles and an intervening marker gene. These genes are alternatively flanked by the recombination target sites bacteriophage *loxP* and yeast FRT. As usual, transformants are selected by the presence of the marker. With the transformed strains, subsequent crosses are used to selectively excise alleles, along with the marker; selection is based on reversion of the marker phenotype. The *loxP* sites produce excision recombination of one allele and the marker sequences when the phage protein Cre is crossed through the strain. An excision between the FRT sites occurs when yeast FLP is crossed through the strain, thereby removing the other allele and the marker. As a result, perfectly homologous strains can be generated with stable, site-matched transgenes. This allows the study of allelic effects without complications of uncontrolled differences with the control strain, and it generates true allelism of the transgenes that can be used to study gene frequency changes in population cages. The technique of allelic coplacement, however, is limited to produce zygotically expressed variation. To control the ontogenic stage of transgene expression, Sun and Tower (1999) extended to whole flies an induced expression system developed for clonal lineages of *Drosophila* cells (Struhl and Basler 1993;

Basler and Struhl 1994). The FLP-OUT technique manipulates the removal of a stop sequence embedded in the transgene promoter. The stop sequence is flanked by FRT elements, and an FLP recombinase gene is elsewhere under the control of an inducible promoter. When FLP recombinase is expressed in cells, the stop is excised and the transgene is transcribed. Thus, it is possible to assess a single strain with and without the expression of the transgene. This ensures consistency of background and insert effects. It also adds the ability to initiate constitutive transgene expression at defined life stages.

As the techniques of transgenic manipulation advance, what is revealed about somatic maintenance as an adaptation? At the onset we established a need to demonstrate that the postulated candidate genes underlying somatic maintenance have the potential to affect the phenotype of day-to-day survival, the hallmark of a maintained soma. The analysis presented here shows that transgenic manipulation holds promise for this initial step but that genetic pitfalls can compromise results. This issue complicates how we interpret results for transgenics of *SOD*, *catalase*, and *EF1 α* . One approach is to extensively replicate control and treatment lines. This is feasible provided that the effect of transgenic overexpression on phenotype is large relative that of the mutational variance generated by the inserts. For this reason Kaiser et al. (1997) suggested that at least an 18% difference between control and treatment lines is needed to unambiguously determine the effect of transgenes on life span. On the other hand, metabolic control theory suggests that this goal will often be unrealized—overexpression may have rather small effects on phenotype. An alternative approach is to use molecularly derived controls, as illustrated by allelic series strains produced from FLP-mediated unequal homologous recombination. In itself, this technique may not control for variation among strains due to insert size and selection markers. Further care must be taken, and I have argued that artifacts are not problematic when there is congruence among strains in their gene expression-to-phenotype functions. Recently developed techniques will provide even finer molecularly based controls. Thus, the tools are in place to resolve the technical challenge of providing genetically comparable lines that differ solely in transgene expression. From this point we can robustly address the fundamental issues of whether wild-type levels of somatic maintenance are optimally efficient and which trade-offs mold life history into cohesive adaptations.

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