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Protein synthesis elongation factor EF-1α expression and longevity in *Drosophila melanogaster*

(protein synthesis in aging/EF-1α expression/lifespan)

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ABSTRACT **It has been proposed that the decline in protein synthesis observed in aging organisms may result from a decrease in elongation factor EF-1α. Transgenic *Drosophila melanogaster* flies carrying an additional copy of the EF-1α gene under control of a heat-inducible promoter have an extended lifespan, further indicating that the EF-1α gene may play an important role in determining longevity. To test this hypothesis, we have quantitated EF-1α mRNA, EF-1α protein, and the EF-1α complex-formation activity in these transgenic flies. Furthermore, we have tested whether the transgene construct is functional—i.e., whether transgenic mRNA is induced when flies are grown at higher temperature. The results show that although there is a clear difference in mean lifespan between the EF-1α transgenic (E) flies and the control transgenic (C) flies, E flies do not express more EF-1α protein or mRNA than C flies kept at the same experimental conditions. Although the transgene can be induced when E flies are heat-shocked at 37°C, transgenic mRNA is not detectable in E flies aged at 29°C. In both lines, the loss in catalytic activity with age is the same. We conclude that the E flies examined here do not live longer because of overexpressing the EF-1α gene.**

The lifespan of each species, including man, is different, but genetically defined. According to the program theory of aging, the longevity of animal species, as well as the lifespan of their cells in culture, is determined by genetically controlled processes similar to those that control early development. Several attempts have been made to demonstrate the existence of longevity-determining genes (1–5), but nothing is known about possible targets for such genes at the cellular or at the molecular level. Most higher organisms contain mitotic as well as postmitotic tissues. Basic mechanisms underlying organismic aging should conceivably be active and therefore be susceptible to age-related changes, both in mitotic, proliferating cells and in postmitotic cells. Thus one has to consider both replicative senescence (limited lifespan of mitotic cells) and postreplicative senescence (loss of function in differentiated cells). How universal are age-related changes at the molecular, cellular, and organismic level? Mitotic proliferating cells may have a short lifespan and require active protein and DNA synthesis at each cell cycle. Postreplicative cells retain full metabolic activity and hence still require constant renewal of proteins. Therefore, regulation of protein synthesis can be considered as one key homeostatic function common to all cells.

A decrease in the rate of protein synthesis with advancing age has been observed in many animals species (6–9). The importance of this decrease in protein turnover is still under debate (10). Experiments carried out by Webster (6, 11, 12) suggested that the age-related decline in protein synthesis in *Drosophila melanogaster* might result from decreased transcription of the EF-1α gene. The eukaryotic polypeptide-chain-elongation factor EF-1α is a GTP-binding protein that catalyzes the binding of aminoacyl-tRNA to the ribosome (13). Based on Webster's experiments, Shepherd *et al.* (14) addressed the question of whether expression of the EF-1α gene may be directly involved in determining the lifespan of the fruit fly. They transformed *Drosophila* with a *P*-element vector containing a cDNA copy of the EF-1α gene under control of the inducible *hsp70* (70-kDa heat shock protein) gene promoter. At 25°C the flies carrying the EF-1α *P* element (E flies) lived significantly longer than the flies carrying a control *P* element (C flies). This difference in mean lifespan was increased when flies were kept at 29.5°C. The authors concluded that the heat-induced overexpression of the EF-1α gene led to increased lifespan of the flies and thus that there might be a positive correlation between the expression of the EF-1α gene and longevity. Their results suggested that an important aging control might be exerted at the level of transcription of the EF-1α gene. We have tested this hypothesis by measuring the expression of EF-1α in these transgenic E and C flies both at the mRNA and at the protein level.

MATERIALS AND METHODS

Flies. The transgenic *D. melanogaster* lines constructed by Shepherd *et al.* (14) were used: E flies contain a *P*-element with the EF-1α cDNA under control of the *hsp70* promoter; C flies contain the same *P* element without EF-1α cDNA. Adult male flies were kept under controlled conditions at 25°C or at 29°C (14).

Gene Construction. A 1-kb *Eco*RI fragment was cut out from the *P*-element plasmid pNHTEF (15) containing the *Drosophila* EF-1α cDNA (16) which had been used to construct the E flies (14) and was cloned in both orientations into pBluescript (Stratagene) to yield pBSshEF and pBSshEF/r. The 1-kb fragment contained the *hsp70* promoter (17) plus 400 bp from the 5′ end of the EF-1α cDNA (Fig. 1).

Quantitation of EF-1α mRNA. Total RNA was extracted according to Chomczynski and Sacchi (18). Equal amounts (5 μg) of total RNA were electrophoresed in 1.2% agarose/2.2 M formaldehyde gels and blotted to nylon membranes. These Northern blots were hybridized with ^{32}P-labeled probes for sequences encoding EF-1α and ribosomal protein rp49 (19) and exposed to x-ray films. Intensities of autoradiographic bands were quantitated by densitometer scanning.

Quantitation of EF-1α Protein. Total soluble protein extracts were prepared by homogenizing flies in extraction buffer: 100 mM Tris Cl, pH 7.9/1 mM dithiothreitol/0.1 mM EDTA/2 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$/1 mM phenylmethanesulfonyl fluoride/20% (vol/vol) glycerol (20). The protein concentration of the lysates was determined with the

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Abbreviations: E flies, EF-1α transgenic flies; C flies, control transgenic flies.

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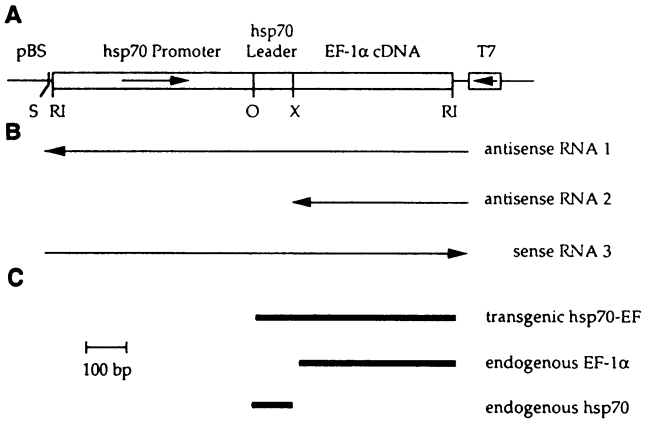

FIG. 1. (*A*) Map of the pBShsEF plasmid (in the plasmid pBShsEF/r the *Eco*RI insert is in the reverse orientation). pBS, pBluescript plasmid sequences (not drawn to scale); T7, T7 promoter sequence; RI, *Eco*RI site; X, *Xba* I site; S, *Sma* I site; O, transcription start site of the *hsp70* promoter. (*B*) Orientation and length of the three RNA probes. Antisense RNA 1 and sense RNA 3 have a length of about 1000 bases; antisense RNA 2 is ≈400 bases long. (*C*) Protected fragments in the S1 nuclease protection assay. Antisense RNA 1 protects 500 bases of the transgene, 400 bases of the endogenous EF-1α mRNA, and 100 bases of the *hsp70* leader of the endogenous *hsp70* mRNA.

Biorad protein assay; equal amounts of protein were electrophoresed in SDS/polyacrylamide gels and blotted to nitrocellulose membranes. Membranes were incubated with a polyclonal rabbit antiserum containing specific antibodies against *Drosophila* EF-1α and actin, followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies (Jackson Laboratories). After developing, the immunoblot signals were quantitated by densitometer scanning.

Quantitation of EF-1α Activity. The formation of stable ternary complexes of EF-1α with ribosomes and charged tRNA was measured according to Slobin and Möller (21) and Merrick (22). Briefly, aliquots from the same protein extracts as described above were incubated with an excess of ribosomes (prepared from *Drosophila* embryos) and [^{14}C]phenylalanyl-tRNA (specific activity, 423 mCi/mmol; 1 mCi = 37 MBq) in the presence of the nonhydrolyzable GTP analog guanosine 5′-[β,γ-imido]triphosphate. The amount of labeled complex retained on nitrocellulose filters was measured in a scintillation counter.

S1 Nuclease Protection Assay. RNA probes—antisense RNA 1, antisense RNA 2, and sense RNA 3 (see Fig. 1)—were *in vitro* transcribed from plasmids pBSshEF and pBSshEF/r in the presence of [α^{32}P-UTP] and phage T7 RNA polymerase (23). Total RNA equivalent to about three flies was used for each protection experiment. Hybridization and S1 nuclease digestion were done as described (24). The products were separated in 5% polyacrylamide gels. After drying, the gels were exposed to x-ray films.

RESULTS

Lifespan Measurements. After having kept the transgenic fly stocks constructed by Shepherd *et al.* (14) for >2 years, we first wanted to confirm whether they still maintained the long-living phenotype. We repeated the lifespan measurements on the transgenic E and C flies at 25°C and at 29°C, using only males. The longevity data presented by Shepherd *et al.* (14) were reproducible: E flies lived longer than C flies, but none of them lived longer than wild-type flies. In our hands, C flies at 25°C had a mean lifespan of 41.3 ± 2.0 days (mean ± SD), and E flies had a mean lifespan of 43.2 ± 1.6 days. Although the absolute lifespan decreased at higher temperature, the *difference* in mean lifespan increased at 29°C: the C flies lived for 24.6 ± 2.8 days and the E flies lived for 29.5 ± 1.7 days. Thus the difference in mean lifespan at 25°C was 4.5%, and at 29°C it increased to 19.8%. By the Welch test, both these differences are statistically significant ($P < 0.0005$). These differences are smaller than those reported by Shepherd *et al.* (14), but it is possible that the fly stocks had changed with time.

Expression of EF-1α mRNA. Total RNA was extracted from aging flies, and the steady-state level of EF-1α mRNA was determined on Northern blots (example in Fig. 2*A*). To be able to compare samples from different fly stocks and different ages, and to compensate for differences in body size, equivalent amounts of total RNA were used. Northern blots were hybridized with probes specific for RNA encoding EF-1α and ribosomal protein rp49. EF-1α and rp49 signals were quantitated on at least two or three Northern blots made from two or three RNA extractions. The signal for rp49 was used as an internal standard and the results are presented as EF-1α/rp49 ratios (Fig. 2*B*). To compare measurements from blots that had been hybridized to probes with different specific activity, the values obtained for young (2-day-old) flies were set at 100%. Comparison of mRNA ratios in E and C flies showed no significant difference between the two lines. At 25°C, the C flies seemed to express slightly more EF-1α mRNA, but the difference lay within the experimental error. At 29°C, both E and C flies expressed approximately the same levels of EF-1α mRNA. We conclude that at the experimental conditions which resulted in an increased lifespan, the E flies do not express significantly more EF-1α mRNA than C flies.

Expression of EF-1α Protein. The amount of EF-1α protein in total soluble protein extracts was determined on Western blots (Fig. 3*A*). Preliminary experiments had shown that the amount of total soluble protein per fly did not change during aging. Equal amounts of total protein were therefore loaded

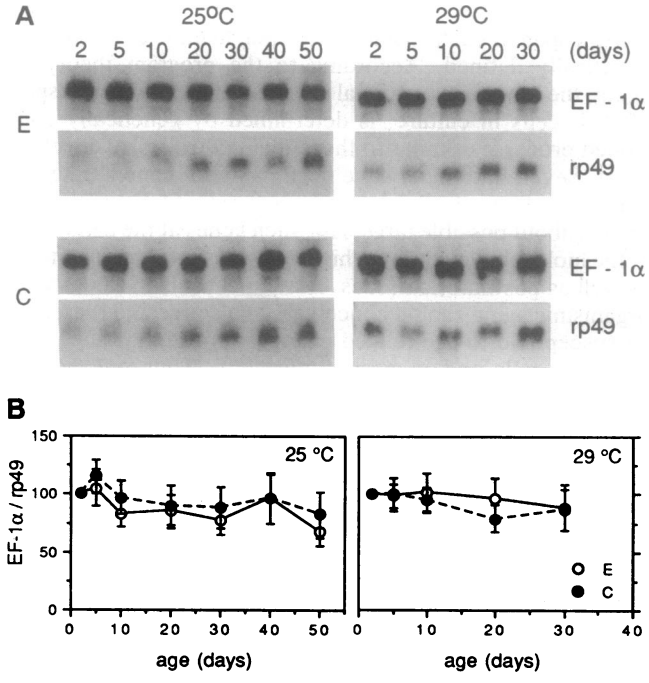

FIG. 2. Comparison of EF-1α mRNA in E and C flies. (*A*) Northern blot. Total RNA was extracted from E and C flies kept at 25°C or 29°C at the ages indicated and electrophoresed in formaldehyde-containing agarose gels. Each lane contained 5 μg of total RNA. The membranes were hybridized with probes for EF-1α and ribosomal protein rp49 and exposed to x-ray film. (*B*) Quantitation of Northern blots. The EF-1α/rp49 ratios of 2-day-old flies were set as 100%. Each point represents the mean ± SD from five or six measurements.

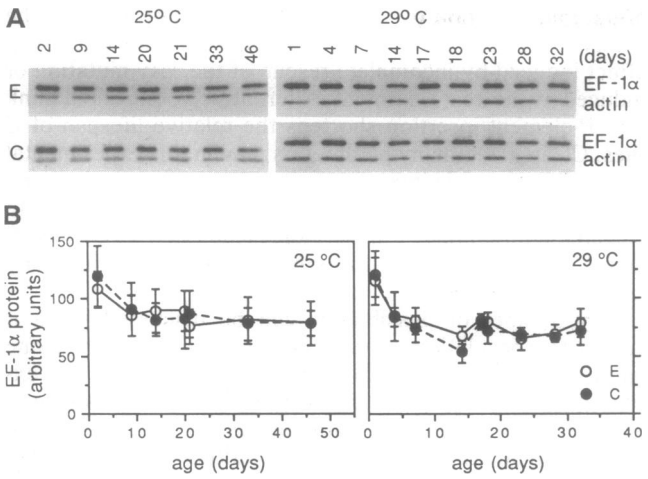

FIG. 3. EF-1α protein in aging flies. (*A*) Immunoblot. Each lane contained 1 μg of total soluble protein extracted from E or C flies of the age indicated grown at the temperature indicated. The rabbit antiserum used recognizes both EF-1α and actin. The actin serves as an internal standard to ascertain that equal amounts of protein were loaded. (*B*) Quantitation of EF-1α protein from immunoblots. Means ± SD from four measurements are given.

in each lane. The immunoblot signals were quantitated by densitometer scanning (Fig. 3*B*). In both lines grown at 25°C we observed a rapid decrease of EF-1α protein between day 1 and day 7 of the adult life and very little change until the end of the lifespan. Similar results were obtained with flies grown at 29°C. In conclusion, comparing absolute amounts of EF-1α protein in E and C flies, we cannot detect a significant difference between the two lines.

EF-1α Activity. There is a possibility that the EF-1α protein becomes inactivated during aging, by changes in posttranslational modification, for example. Small differences in newly synthesized active protein may not be detectable by measuring mRNA or protein levels. For this reason we determined EF-1α activity in the various fly extracts. The amount of active EF-1α was measured by the formation of stable ternary complexes of EF-1α with ribosomes and charged tRNA in the presence of a nonhydrolyzable GTP analog (see *Materials and Methods*). In the presence of an excess of ribosomes and an excess of [^{14}C]phenylalanyl-tRNA, the amount of labeled ternary complex formed is dependent on the amount of active EF-1α. In this assay, crude protein extracts can be used, and we took aliquots from the same protein samples that had been used for the Western blots. Fig. 4 shows the binding activity from E and C flies grown at 25°C and at 29°C: in all fly extracts we observed a similar decrease of EF-1α activity. Again, there was no difference

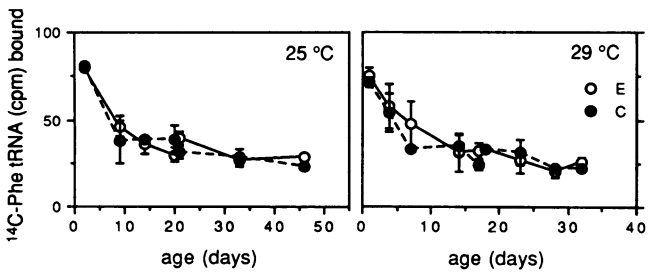

FIG. 4. Changes in EF-1α binding activity during aging. Active EF-1α was determined in aliquots of the total soluble protein extracts used for immunoblots. The assay measures ternary complex formation by EF-1α, charged tRNA and ribosomes. The amount of [^{14}C]phenylalanyl tRNA bound to nitrocellulose filters was measured. Values represent means ± SD of two to four measurements.

between E and C flies. Interestingly, the activity dropped to a much lower value than the EF-1α protein measured on Western blots: about half of the protein detected on immunoblots was no longer active in the old flies. This was true for both lines at all conditions tested. We conclude that during senescence, EF-1α protein loses catalytic activity, possibly by changes in posttranslational modifications.

At this point we had to conclude that there were no striking differences in EF-1α mRNA level, EF-1α protein content, or EF-1α activity between the E and C flies. Because of the small size difference, we cannot distinguish between endogenous and transgenic EF-1α mRNA on the Northern blots. The question then arises whether the transgene can be induced at all. With a nuclease protection experiment we tested whether the transgene was induced at elevated temperature. In addition, the half-life of the transgenic EF-1α mRNAs was measured.

Inducibility of the Transgene. To test for the inducibility of the transgene, labeled sense and antisense probes of different length (Fig. 1) were generated by *in vitro* transcription and used in RNase protection assays. The antisense RNA 1 contained 95 bases of the *hsp70* leader and 400 bases of the EF-1α cDNA, whereas the antisense RNA 2 contained only the 400 bases of the EF-1α cDNA. Thus, the antisense RNA 1 probe allowed us to clearly distinguish between transgenic and endogenous EF-1α message. The sense-strand (RNA 3) probe was used as a negative control.

Fig. 5*A* shows an S1 nuclease protection assay in which total RNA extracted from E flies grown at 25°C and 29°C and from E and C flies heat-shocked for 1 hr at 37°C had been hybridized to the three RNA probes. With the specific antisense RNA 1 probe the transgenic message could be detected only in E flies heat-shocked at 37°C, but not in E flies grown at 25°C or 29°C or in heat-shocked C flies. A strong signal of the endogenous EF-1α mRNA was present in all samples, detected with probes 1 and 2. The RNA 1 probe also allowed us to detect the endogenous *hsp70* mRNA. This experiment shows that the endogenous *hsp70* promoter was induced weakly at 29°C and very strongly at 37°C, whereas the transgenic *hsp70*–EF-1α gene was not induced at 29°C. In the second experiment, E flies were heat-shocked for 1 hr at 29°C and 33°C. Transgenic EF-1α mRNA was induced only weakly at 33°C, whereas the endogenous *hsp70* gene was strongly induced at 33°C (Fig. 5*B*).

These experiments clearly show that the construct is functional in the transgenic E flies. One might argue that transgenic message is made in very small amounts and has a turnover too short to be detected. This question was addressed by determining the half-life of the transgenic mRNA.

Half-Life of the Transgenic mRNA. E flies growing at 25°C were heat-shocked for 1 hr at 37°C and then placed back at 25°C. Total RNA was extracted at 0, 1, 2, and 4 hr after heat shock and used for S1 nuclease protection assays as described above (Fig. 5*C*). The signals from the transgenic and the endogenous protected EF-1α mRNAs were quantitated by densitometer scanning. The inducible transgenic mRNA had a half-life of ≈2 hr as calculated from the densitometric measurements. The endogenous EF-1α mRNA signal was used as an internal standard, under the assumption that the amount of endogenous EF-1α mRNA would stay constant during heat shock treatment.

DISCUSSION

This paper shows that the transgenic EF-1α flies do not express measurable amounts of transgenic mRNA, nor do they contain higher amounts of EF-1α protein or higher EF-1α activity than control flies. This is true for flies grown at 25°C and at 29°C. This result was unexpected. It had been postulated that the *hsp70* promoter would drive transcription

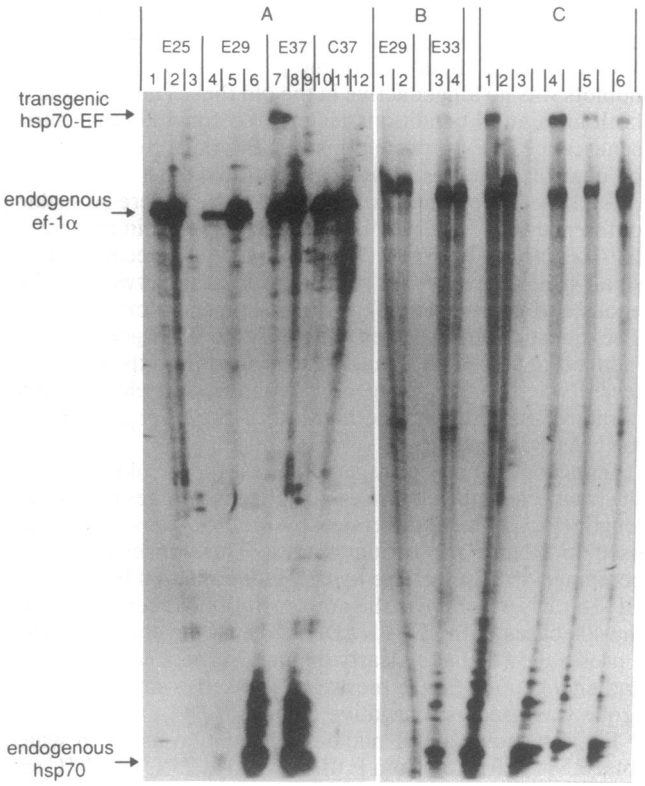

FIG. 5. Inducibility and half-life of the transgene. Autoradiograph of gels from S1 nuclease protection assays. (*A*) Total RNA was extracted from flies grown at 25°C or 29°C (lanes 1–6) or heat-shocked at 37°C for 1 hr (lanes 7–12) and was probed with antisense RNA 1 (lanes 1, 4, 7, and 10), antisense RNA 2 (lanes 2, 5, 8, and 11), or sense RNA 3 (lanes 3, 6, 9, and 12). Protected fragments corresponding to endogenous EF-1α mRNA, transgenic EF-1α mRNA, and *hsp70* mRNA are indicated by arrows. Transgenic message was induced only in E flies at 37°C (lane 7). (*B*) Total RNA from E flies that were heat-shocked for 1 hr at 29°C or 33°C was probed with antisense RNA 1 (lanes 1 and 3) or antisense RNA 2 (lanes 2 and 4). The transgene was induced weakly at 33°C but not at 29°C. (*C*) Stability of the transgenic mRNA. E flies were heat-shocked for 1 hr at 37°C and then placed back at 25°C. RNA was extracted at time 0 (lanes 1–3) and after 1, 2, and 4 hr (lanes 4–6), and probed with antisense RNA 1 (lanes 1 and 4–6), antisense RNA 2 (lane 2), and sense RNA 3 (lane 3).

from the transgene at 29°C, because it did induce transcription from a white (*w*) transgene even at low temperature (17). As the heat-shock experiments clearly demonstrate, the construct introduced into the flies is functional; i.e., it can be induced weakly at 33°C and strongly at 37°C. However, it is not induced when flies are grown at 29°C. The lack of transgenic mRNA cannot be explained by a rapid turnover: the S1 nuclease protection experiment shows that the half-life of induced transgenic mRNA is ≈2 hr, which should be long enough to be functional. In conclusion, we can say that these E flies do not live longer because of overexpression of the transgene.

Why then do the E flies live longer than the C flies? Several possibilities can be discussed. First one has to reconsider the original lifespan data by Shepherd *et al.* (14), which are reproducible in our hands. The extension of the mean lifespan of E flies is only *relative* to C flies at the experimental conditions used. It must be pointed out that both the rosy mutation and the rise in temperature have dramatic effects on the lifespan of fruit flies. Rescue of the rosy phenotype by the *P*-element vector used in Shepherd's flies did not restore the normal lifespan: in our hands none of the transgenic flies lived as long as wild-type flies, either at 25°C or at 29°C. One might argue that insertion of the transgene does not increase the lifespan of E flies *per se*, but that it reverts a life-shortening effect induced by incomplete rescue of the rosy mutation or induced by the heat treatment. Alternatively, one could think that *P*-element insertion shortens the relative lifespan of C flies. Clearly, too many parameters play in this experimental setup: differences in integration site of the transgene in E and C flies (position effect), as well as heat-shock effect due to elevated temperature. Furthermore, using only one single E and C line each, one is certainly not allowed to draw any general conclusions.

Any number of possibilities can be discussed when it comes to speculations about position effects. On the one hand, insertion position can significantly influence the expression of a transgene (25, 26). On the other hand, *P*-element insertion may result in disruption of a gene that may be involved in determining lifespan. Mutations affecting longevity have been discovered in *Caenorhabditis elegans*: e.g., the gene *age-1* increases the lifespan of the worm (4, 5). Because none of our transgenic flies live longer than wild-type flies, we can at least rule out the possibility that the *P* element has hit a longevity-determining gene analogous to *age-1*.

The question of position effect and genetic background effect on lifespan and fitness components of *D. melanogaster* has been addressed in detailed studies by Stearns *et al.* (27) and Stearns and Kaiser (28). Starting with the E and C stocks constructed by Shepherd *et al.* (14), Stearns and coworkers demonstrated that crossing these flies to different genetic background or changing the EF-1α insert position on the third chromosome had dramatic effects on the lifespan of both the E- and the C-derived lines. They concluded that the effects of genetic background and chromosomal position on lifespan were much more important than the effect of EF-1α overexpression. Although these experiments clearly point out the importance of the problem, they do not address the question of whether any of the lines used in this study do express the transgene. It is likely that similar biological effects may have resulted from changing the genetic background and/or the position of any DNA insert and has nothing to do with EF-1α.

The hypothesis proposed by Shepherd *et al.* (14) that increased EF-1α gene transcription may influence the lifespan of *Drosophila* has by now been widely accepted or heavily debated (27, 28). In both cases it was assumed that the transgene was overexpressed. Although our results cannot confirm that the particular transgenic E line examined overexpresses EF-1α, they do not yet disprove the hypothesis. From our Northern blots it may appear that both EF-1α and rp49 signals increase slightly in aging flies. This may be misleading, because we have loaded equal amounts of total RNA from each fly extract. However, only recently, an age-related decline in the rRNAs has been detected (N.S., unpublished results) which would result in a decrease of total RNA in old flies. Furthermore, there is evidence that many ribosomal protein genes are downregulated in senescent cells (29). Therefore, the EF-1α/rp49 ratios do not tell us anything about postulated changes in EF-1α mRNA levels during aging; they only allow us to compare levels in E and C flies. Experiments in other systems support the idea that EF-1α activity is controlled at the transcriptional level. A decrease in EF-1α mRNA was observed in aging rats (6) and in differentiating mouse erythroleukemia cells (30). Cavallius *et al.* (31) observed that both EF-1α protein and EF-1α catalytic activity decreased in aging fibroblasts but remained constant in simian virus 40-transformed "immortal" fibroblasts. In addition, there is evidence that EF-1α may be regulated at the translational level (32) or by changes in posttranslational modification (33). Recently it was shown that mutations in EF-1α resulted in increased lifespan of filamentous fungi by influencing the translational fidelity (34).

Here we show that the amount of EF-1α protein in senescent E and C flies decreases only slightly, whereas the catalytic activity drops to about 30%. The same values were also obtained when EF-1α activity was measured in wild-type *Drosophila* (N.S., unpublished results). Recently, several authors have reported changes in protein activity in aging cells or organisms—e.g., in various enzymes (reviewed in ref. 10), or in the heat-shock transcription factor, HSTF (A. Richardson, personal communication). Loss of enzyme function seems then to be a general phenomenon in aging organisms. EF-1α is a very abundant protein in all cells and may be involved in other cellular functions. Our data cannot rule out that the fraction of EF-1α protein which is no longer active in protein synthesis plays another, perhaps structural, role in aging flies. EF-1α has been found in association with cytoskeletal elements (36, 37), as well as with membranes and with the mitotic apparatus (35, 38, 39). Age-related changes in any of these structures may also have deleterious effects on cell function. It would be interesting to see whether any of the long-living lines of *D. melanogaster* which have been obtained by natural selection of late-reproducing females (1–3) or the *age-1* mutant of *C. elegans* (4, 5) express elevated levels of EF-1α.

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