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Genetics

Overexpression of Cu–Zn superoxide dismutase in *Drosophila* does not affect life-span

(aging/free radicals/paraquat/P element transformation/transgenic *Drosophila*)

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ABSTRACT **Aging and disease processes may be due to deleterious and irreversible changes produced by free radical reactions. The enzyme copper–zinc superoxide dismutase (Cu–Zn SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) performs a protective function by scavenging superoxide radicals. The Cu–Zn SOD gene (*Sod*) cloned from *Drosophila melanogaster* was introduced via P element-mediated transformation into the germ line. Homozygous lines carrying additional copies of the *Sod* gene were recovered and characterized. Increases in *Sod* transcripts and enzyme activity were observed in the transformed lines, indicating that all of the sequence information required for gene expression is contained on the inserted gene fragment. The effects of additional SOD on oxygen free radical metabolism and longevity were investigated. Additional SOD did not markedly affect oxygen metabolism or longevity.**

Aging and certain disease processes may be due to the cumulative effects of deleterious and irreversible changes produced by free radical reactions. The free radical theory of aging is supported by the observed inverse relationship between the average life-span of mammals and their basal metabolic rates and by the fact that antioxidants can increase the average life-span of mice (1). This theory predicts that a normal life-span may be extended by increasing antioxidant levels to a point where they minimize deleterious free radical reactions.

In aerobic organisms the metabolism of oxygen is a principal source of free radicals as a small fraction of molecular oxygen is converted to superoxide (O_2^{-}) (2). A primary defense against these deleterious free radicals is the enzyme copper–zinc superoxide dismutase (Cu–Zn SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1), which scavenges superoxide by dismutating it to hydrogen peroxide (H_2O_2) and molecular oxygen (3). Removal of the superoxide radical by SOD and of hydrogen peroxide by catalase and glutathione peroxidase prevents formation of the very reactive hydroxyl radical ($^{\cdot}OH$), which is postulated to be responsible for much of the cellular damage. Reduction of the concentration of the free radical by SOD in cells may reduce the incidence of cancer and other diseases, as well as increase life-span.

We have chosen the fruit fly, *Drosophila melanogaster*, as a model organism for studying the effects of free radical metabolism on aging. Not only does *Drosophila* have a short life-span, but it also possesses a wealth of genetic information. Furthermore, the postmitotic nature of most of the somatic tissues of the adult favors studies of aging processes (4).

In higher vertebrates, there is a direct correlation between longevity and the ratio of SOD levels to specific metabolic rates (5). In poikilotherms, an inverse relationship between metabolic rate and life-span has been demonstrated (6). More specifically, in *Drosophila*, the superoxide scavenging capacity of the tissues and the respiration rate are important determinants of life-span (7). However, the role of SOD in *Drosophila* aging is still unclear since two different wild-type strains with the same level of SOD activity have been found, yet their life-spans differ by 40% (8). In light of these divergent results, the genetic background of *Drosophila* must be controlled in order to study unambiguously the effects of different levels of SOD on aging.

To determine whether additional SOD activity affects oxygen metabolism and longevity in *Drosophila*, we cloned the *Sod* gene from an isogenic stock and introduced additional copies of the gene back into the genome of the same stock via P element-mediated transformation (9, 10). In *Drosophila*, P elements are efficient vectors for the introduction of defined segments of DNA into germ-line cells. Subsequent expression of the introduced gene may be indistinguishable from that of the endogenous chromosomal gene. The *Sod* gene from *D. melanogaster*, which has been cloned and analyzed (11–13), was recloned into the P element vector pUChsneo (14).

Using this approach, we have constructed a series of *Drosophila* strains, each of which contains an additional copy of the *Sod* gene at a different chromosomal site. Analysis of these transformants indicates that sufficient sequence information required for the functional expression of the *Sod* gene is contained within the inserted gene fragment. In this paper, we present an analysis on the effects of different levels of SOD gene expression on oxygen free radical metabolism and life-span in the transformants as well as in flies heterozygous for the SOD deficiency, *Df*(3L)lxd^9/Tm3*SbSer* (15) and in flies homozygous for a SOD "null" mutant (16).

MATERIALS AND METHODS

***Drosophila* Stocks.** The recipient strain used for P element transformation by microinjection was wild-type (wt) isogenic Oregon R (G.M.T., unpublished data). The *Sod* null mutant strain was generously provide by F. J. Ayala (University of California, Irvine). The chromosome-2, -3 balancer stock *CyO*;TM2,*Ubx*/T(2;3)ap^{Xa} (17) was obtained from P. L. Davies (Queen's University, Kingston, ON, Canada). The *Sod* deficiency strain *Df*(3L)lxd^9/TM3*SbSer* was kindly provided by E. M. Meyerwitz (California Institute of Technology, Pasadena) and V. Finnerty (Emory University, Atlanta).

P Element-Mediated Transformation. The 1.8-kilobase (kb) *Eco*RI restriction fragment containing the *Sod* gene was isolated (12, 13) and ligated into the unique *Eco*RI restriction site of the P element plasmid pUChsneo (14) by standard techniques (18). Microinjection of *Drosophila* embryos was carried out essentially as described (9, 10). Transformants were selected on food containing the antibiotic G418 (14). The chromosome-2, -3 balancer *CyO*;TM2,*Ubx*/T(2;3)ap^{Xa} was used to obtain flies homozygous for the inserted trans-

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poson. The resulting transformed lines have the X, second, and third chromosomes from the recipient wt isogenic strain. Therefore, the genetic background of the homozygotes is identical to that of the wt isogenic strain except for the Y chromosome, which comes from the double balancer stock.

***In Situ* Hybridization to *Drosophila* Polytene Chromosomes.** The polytene chromosomes from the transformed lines were prepared for *in situ* hybridization (19) and, in addition, were acetylated to decrease nonspecific binding of the probe (20). The *Sod* gene probe (subcloned in the plasmid pUC13) was labeled by nick-translation with biotin-substituted 11-dUTP (21) and the resulting hybridization signals were detected by a streptavidin-alkaline phosphatase detection system (BluGENE kit; BRL).

Northern and Southern Blot Analysis. *Drosophila* DNA was prepared from adults by the method of Jowett (22). Restriction enzyme digests of 5 μg of genomic DNA were separated by electrophoresis on 0.7% agarose gels. The DNA was then transferred to nylon membranes (Hybond-N, Amersham) by the method of Southern (23). Total *Drosophila* RNA was purified by the guanidinium thiocyanate/CsCl method essentially as described (24). Total RNA separated by electrophoresis on 1.4% agarose gels containing 0.66 M formaldehyde was transferred onto nylon membranes after the gels were saturated with 10× SSC (1× SSC = 0.15 M NaCl/0.015 M trisodium citrate, pH 7.0). The RNA on the membranes was hybridized with radiolabeled DNA probes and exposed to film. The autoradiogram was used to determine the regions of the filter containing *Sod*-specific RNA. These regions were cut out and the radioactivity of each piece was determined in a scintillation counter by Cerenkov radiation emitted in the presence of water. The average background on the membrane, determined by analyzing areas equal in size to the region containing *Sod* RNA from above and below these regions, was subtracted from the radioactivity of the *Sod* RNA regions. The levels of *Sod*-specific mRNA were standardized by comparison to levels of three actin transcripts, which were quantified in the same manner. These standardized values from the transformants and the heterozygous SOD deficiency *Df*(3L)lxd^9/TM3*SbSer* were compared to that of wt.

Enzymatic Assays of SOD Specific Activity. For each assay, five male or female adult flies (<24 hr old) from each line were homogenized in 800 μl of 50 mM potassium phosphate, pH 7.8/0.1 mM EDTA buffer. The SOD activity in the homogenates was determined by a cytochrome *c* spectrophotometric assay at pH 10 as described (25). The protein concentration of the extract (26) was used to determine the SOD specific activity. In each transformed line and in the heterozygous SOD deficiency, *Df*(3L)lxd^9/TM3*SbSer*, the SOD specific activity in males and in females was compared to that of their wt counterparts. Results from multiple assays made over several generations were averaged.

Assay of Paraquat Toxicity. Adults (0–2 days old) were separated by sex and exposed to paraquat for 48 hr at 25°C in vials (20 per vial) containing filter paper saturated with 0–40 mM paraquat in 1% sucrose solution. The number of living flies was determined after 48 hr and this was expressed as a percentage of the total starting number.

Life-Span Determinations. The SOD null mutant (16), heterozygous *Sod* deficiency *Df*(3L)lxd^9/TM3*SbSer* (15) and isogenic Oregon R were crossed to produce the SOD null/wt, SOD null/*Df*(3L)lxd^9, and *Df*(3L)lxd^9/wt hybrids used in the aging studies. For all the strains studied, including the transformants, newly eclosed adults were collected at 0–24 hr of age, separated by sex, and placed into 8-dram shell vials (10 per vial). The adults were maintained at 25°C or 29°C and serially transferred to vials with fresh medium every 2 days. To obtain survival curves, the number of living flies was determined for each genotype at each transfer and this was expressed as a percentage of the total (27).

RESULTS

P Element-Mediated Transformation. The 1.8-kb gene fragment cloned into pUChsneo contains the coding region of the *Sod^F* gene [a fast electrophoretic variant of SOD (28, 29)] as well as 413 base pairs (bp) of the 5′ untranslated region, 247 bp of the 3′ flanking DNA, and the single 725-bp intron (12). The putative transcriptional control signals in the 345 bp 5′ of the transcription start site have been described (13). Germline transformants were selected on the basis of acquired resistance to G418. From 925 injected embryos, 104 fertile adults were obtained, of which five produced progeny resistant to G418. These resistant (G1) flies were used to establish five independent transformed lines.

The chromosomal positions of the transduced *Sod* DNA sequences were determined by *in situ* hybridization of a *Sod* gene probe to salivary gland polytene chromosomes of larvae from the transformed lines. In each transformed line, only one other site of hybridization was observed besides that of the endogenous gene at 68A4-9 (13). Of the five lines examined, four had inserts on chromosome 2R at 48B, 49BC, 51AB, and 60B, while the fifth line had an insert on chromosome 3L at 67AB (Table 1).

The transformed lines were made homozygous by the use of balancer chromosomes (see *Materials and Methods*). The transposed *Sod* gene was crossed back into the original isogenic recipient in order to maintain a uniform genetic background. Four of the five transformed lines were made

Table 1. SOD specific activity and transcripts in transformed and control strains

Strain	No. of genes	Site of insert	SOD specific activity		SOD transcript
			Female	Male	
Df(3L)lxd^9	1		66 ± 15 (5)	60 ± 7 (6)	50 ± 7 (4)
wt	2		100	100	100
Sod$^+$-4	3	49BC	145 ± 18 (6)	132 ± 12 (6)	149 ± 39 (5)
Sod$^+$-1	4	48B	131 ± 16 (6)	134 ± 10 (6)	99 ± 8 (4)
Sod$^+$-2	4	60B	153 ± 19 (8)	142 ± 2 (5)	153 ± 42 (5)
Sod$^+$-3	4	67AB	164 ± 26 (10)	146 ± 19 (10)	137 ± 15 (5)
Sod$^+$-5	4	51AB	167 ± 25 (6)	170 ± 22 (8)	130 ± 16 (4)

The number of *Sod* genes in the transformed and control strains and the chromosomal site of the transposed *Sod* gene in the transformed strains are shown. The SOD activity in a homogenate of adult males and of females of each strain was normalized to the amount of protein present. The number reported is the mean percentage of the SOD activity in each strain normalized to that of wild-type (wt) *Drosophila*. Total *Drosophila* RNA was prepared from males and females as described. The *Sod* mRNA was normalized to the amount of actin transcript present in each sample. The number reported is the level of *Sod* mRNA present in each strain expressed as a percentage of the wt level. Means ± SD are given. Numbers in parentheses record the number of individual determinations.

isogenic and homozygous for the single transposon insertions. These flies would have four potentially functional *Sod* genes (Table 1). We were unsuccessful in getting a homozygote for the Sod^+*-4* line, which has a transposon inserted at 49BC on chromosome 2R. Presumably, this insert disrupts an essential gene, making the homozygous state lethal. Further analysis of this transformed line with three potentially functional *Sod* genes was carried out on heterozygotes in which the chromosome carrying the *Sod* gene was maintained over the second chromosome balancer, *CyO*. This Sod^+*-4* line is isogenic with the other transformed lines except for the second chromosome.

The transduced *Sod* DNA sequences were analyzed by a Southern blot of genomic DNA from each transformed line. As expected, hybridization of the *Sod* gene to genomic DNA digested with *Eco*RI revealed an additional single copy of the *Sod* gene in each transformant (Fig. 1). The genomic DNAs from the transformed lines were analyzed after many generations of inbreeding. Thus, the transduced gene appears to be stably integrated (9, 10).

Expression of the Transduced *Sod* Gene. Northern analysis revealed that the transduced *Sod* gene was expressed and *Sod* mRNA of the correct size was produced in each transformed line (Fig. 2). The level of *Sod*-specific mRNA was standardized relative to three actin transcripts (30), which are unaffected by the transformations. After comparing the transformants to the wt recipient strain (with only two endogenous *Sod* genes), it is evident that in all cases, except in Sod^+*-1*, the SOD mRNA levels in the transformants were higher (Table 1). In contrast, a heterozygous deficiency of the *Sod* region, *Df*(3L)lxd^9/TM3*SbSer* (*Df* 68A3,4–68B4,C1) (15), with only one copy of the *Sod* gene, produced 50% of the *Sod* transcript relative to wt. Differences in *Sod* expression between the transformed lines may be attributed to the different chromosomal positions of the inserted gene in each case.

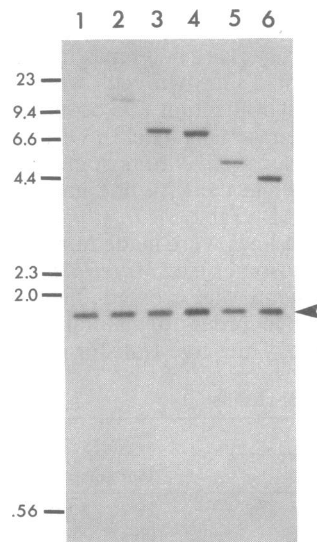

FIG. 1. Analysis of the DNA from transformed flies by Southern blot analysis. Genomic DNA from adult flies of the wt recipient strain (lane 1) and five transformed lines (lanes 2–6, Sod^+*-1* to Sod^+*-5*) was digested with *Eco*RI restriction endonuclease, separated by electrophoresis in a 0.7% agarose gel, and transferred to a nylon membrane. A pUC13 plasmid containing the 1.8-kb *Sod* gene fragment was radiolabeled and hybridized to DNA on the membrane. The resulting autoradiogram is shown. The 1.8-kb hybridizing band represents the endogenous *Sod* gene as well as the transposed *Sod* gene. The five transformed lines have an additional hybridizing band, which is the result of a fusion of the genomic DNA sequence with the remaining part of the pUChsneo vector. Size markers were from a *Hin*dIII digest of λ DNA run in a parallel lane (sizes shown are in kb).

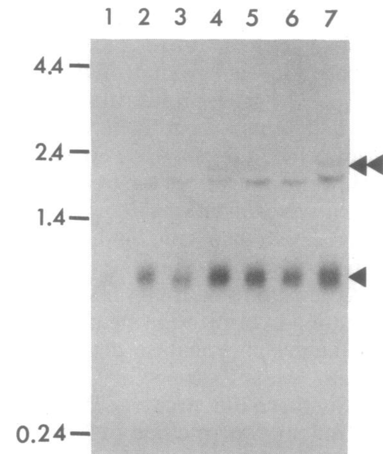

FIG. 2. Northern blot analysis of endogenous and introduced *Sod* genes. Total RNA (30 μg) from each *Drosophila* strain was separated by electrophoresis in a 1.4% agarose gel containing formaldehyde and transferred to a nylon membrane. The membrane was hybridized with radiolabeled plasmids containing the *Sod* cDNA and the actin gene. The resulting autoradiogram shows all strains have a single *Sod* transcript at 0.7–0.8 kb (arrowhead) and the three actin transcripts (double arrowhead). Lanes 1–7, RNAs from *Sod* deficiency *Df*(3L)lxd^9/TM3*SbSer*, wt Oregon R, and transformant lines Sod^+*-1* to Sod^+*-5*, respectively. An RNA ladder (BRL) was used for size markers (in kb). The regions corresponding to the *Sod* and actin gene transcripts were excised from the membrane and the transcripts were quantified by scintillation counting of the emitted Cerenkov radiation.

SOD Specific Activity in the Transformed Lines. Since the *Sod* gene is expected to be expressed constitutively in all tissues, the specific activity measurements were performed by using homogenates of whole flies. The range of SOD activity for the five transformed lines was 131–170% of the value for the wt (Table 1). These results of experiments repeated on flies from subsequent generations demonstrated that the increased specific activity was a heritable trait. In contrast, the heterozygous SOD deficiency *Df*(3L)lxd^9/TM3*SbSer* was found to have 60% of the wt SOD activity. There was a good correlation between the amount of *Sod* transcript and the level of SOD activity in the strains studied.

Sensitivity to Paraquat Toxicity. The herbicide paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; Pq^{2+}) generates O_2^- radicals *in vivo* by a mechanism that involves the NADPH-dependent reduction of Pq^{2+} to the relatively stable Pq^+ radical, which reacts rapidly with O_2 to produce O_2^- (31, 32). It is also highly cytotoxic and lethal to animals. Feeding adult *Drosophila* with aqueous Pq^{2+} may result in the exposure of the fly to concentrations of O_2^- radicals above the tolerance level of the fly's endogenous protective mechanisms. Since the results of the assays for male and female flies were similar, the data were pooled. The SOD null mutant with only 3.5% of the wt level of SOD protein (16) is clearly hypersensitive to Pq^{2+} exposure due to its decreased capacity to dismutate O_2^- (Fig. 3). The LD_{50} values were 2 mM for the SOD null mutant and 10 mM for wt isogenic Oregon R flies. The *Df*(3L)lxd^9/TM3*SbSer* strain has ≈60% of the wt SOD protein but showed a Pq^{2+} sensitivity comparable to that of wt (Fig. 3). The transformed lines overexpressing SOD showed no increased resistance to paraquat. In fact, their LD_{50} values were either comparable to wt or lower (Fig. 4).

Life-Span Determinations. Since the superoxide scavenging ability of tissues has been implicated as an important determinant of life-span (1, 5, 7), we investigated the life-span of the SOD null mutant as well as that of the transformed lines overexpressing SOD. At 29°C, the SOD null mutant had a

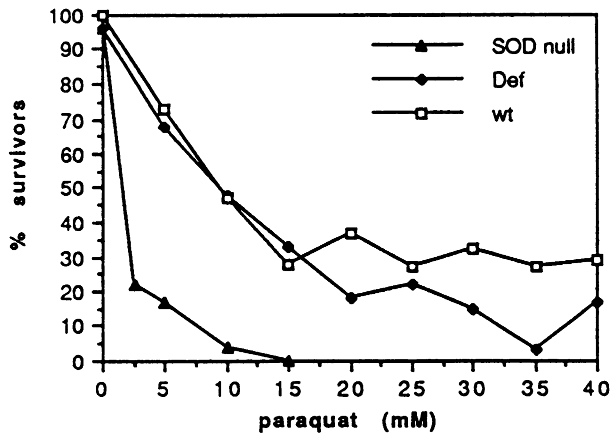

FIG. 3. The sensitivity of the SOD null and *Df*(3L)lxd^9/TM3*SbSer* strains to paraquat exposure for 48 hr at 25°C. The SOD null was hypersensitive to paraquat. The response of the SOD deficiency *Df*(3L)lxd^9 to paraquat exposure was similar to that of wt. Each data point represents the analysis of 100–300 males and females.

mean adult life-span of 28 days compared to 42 days for wt (Fig. 5). Since the genetic background of the SOD null mutant differed from that of the isogenic wt strain, we determined the mean life-spans of *Df*(3L)lxd^9/SOD null, SOD null/wt, and *Df*(3L)lxd^9/wt flies. These were 46, 52, and 56 days, respectively (data not shown).

Finally, the transformed lines overexpressing SOD had mean life-spans comparable to wt. At 29°C, males from the transformed lines *Sod*$^+$*-1*, *Sod*$^+$*-2*, *Sod*$^+$*-4*, and *Sod*$^+$*-5* had mean life-spans of 39, 44, 43, and 42 days, respectively. Similar trends were observed for female flies. Only the data for *Sod*$^+$*-4* and *Sod*$^+$*-5* males is shown (Fig. 5). Similarly, at 25°C, the mean life-spans of all the transformed lines were comparable to the wt value of 61 days.

DISCUSSION

In this study, transformed lines were successfully created with additional copies of the SOD gene. Despite the small amount of DNA flanking the gene used in the transformation, increased *Sod* expression and enzyme activity was obtained. Therefore, the DNA used in the transformation includes all the cis-acting sequences necessary for the *in vivo* expression of the *Sod* gene. Since the transformants have the same

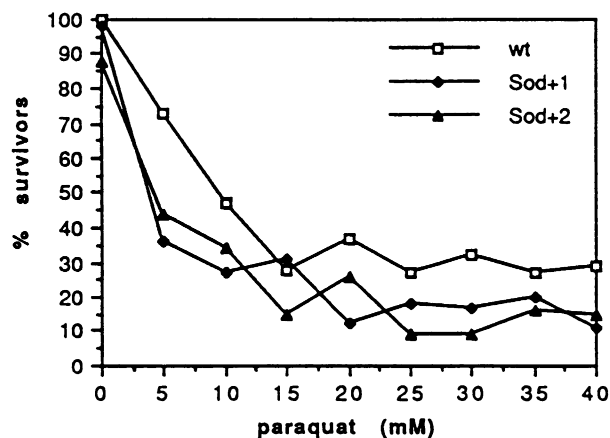

FIG. 4. The sensitivity of the *Sod* transformants to paraquat exposure for 48 hr at 25°C. The response of all transformed lines was similar or more sensitive than wt. Representative transformant lines are shown. Each data point represents the analysis of 100–300 males and females.

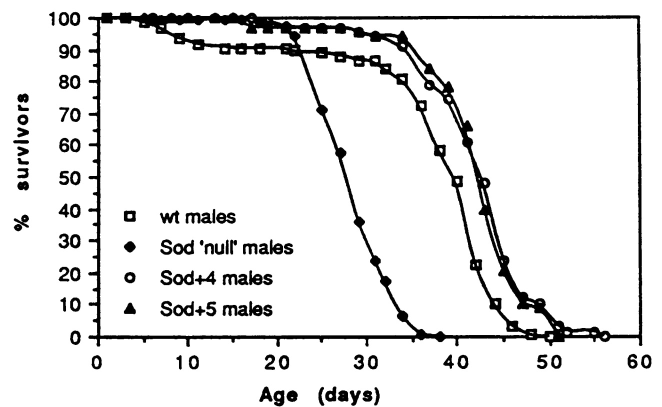

FIG. 5. Longevity determination for SOD null and *Sod* transformants at 29°C. The SOD null strain has a decreased mean adult life-span but the additional SOD activity in the transformants does not affect adult life-span compared to isogenic wt Oregon R. The male survival curves from representative transformed lines are shown. In each strain, 70–200 males were analyzed.

genetic background as the wt controls, it was now possible to compare unequivocally the effect of increased SOD activity on oxygen metabolism and longevity in *Drosophila*. We found that increased SOD levels have little effect on resistance to paraquat-generated O_2^- and life-span and that very low SOD levels provide significant protection against O_2^- cytotoxicity.

Increased SOD activity in *Drosophila* did not confer greater resistance to paraquat-generated superoxide radicals. On the contrary, in some transformants, increased activity resulted in a greater sensitivity to paraquat. It may be argued that with elevated levels of SOD activity, the O_2^- generated by Pq^{2+} would be dismutated to H_2O_2 as well as to other active forms of oxygen ($^{\bullet}OH$,$^1O_2$), which may be more toxic than O_2^- itself. In cultured mammalian cells, clones that overproduced SOD showed increased lipid peroxidation when exposed to paraquat (33). Also, lipid peroxidation in biological systems has been implicated in various pathological conditions (34). Likewise, *Escherichia coli* that overproduces Fe SOD (35, 36) and Mn SOD (36, 37) was more sensitive than wt to paraquat. Thus, elevated levels of SOD activity alone are not necessarily advantageous.

Although increased SOD activity is not essential, a threshold concentration of this enzyme must be present to overcome paraquat toxicity. The SOD null mutant with 3.5% of the wt SOD activity was clearly hypersensitive to paraquat-generated O_2^-. This partial SOD null had a LD_{50} value of 2 mM for exposure to paraquat, while the mutant *cSOD*^{n108}, which is apparently devoid of SOD activity, had a much lower value of 0.05 mM (38). In contrast, the heterozygous SOD deficiency *Df*(3L)lxd^9/TM3*SbSer* with 50% of the wt SOD activity was not hypersensitive to O_2^- generated by paraquat. This result suggests that 50% of the wt SOD activity provides sufficient protection against O_2^- cytotoxicity.

Catalase also plays an important role in cellular defense against oxygen toxicity by removing the H_2O_2 formed by SOD. Parallel to the findings with SOD, a mutant, *Cat*^{n2}, with only 5% of the wt catalase activity exhibited a 2-fold increase in resistance to H_2O_2 when compared to the *Cat*^{n1} mutant that has no detectable catalase activity (39). Also, a 50% reduction in catalase activity had little or no effect on H_2O_2 sensitivity (39). Thus, mutants lacking SOD or catalase activity are dramatically impaired in oxygen metabolism, those with a few percent of the wt activities are provided with significant protection against O_2^- and H_2O_2 toxicity, while those with 50% of the wt levels are as resistant as wt.

The free radical theory of aging would predict that a SOD null mutant with its low SOD level and subsequent impaired oxygen defense system would have a reduced life-span. In fact, the life-span of the SOD null mutant was found to be reduced when compared to wt isogenic Oregon R, which had a different genetic background. In contrast, the *Df*(3L)lxd^9/SOD null hybrid, which had undetectable levels of SOD that are nevertheless expected to be lower than that in the homozygous SOD null, has a mean life-span exceeding that of wt. This unexpected finding may be a reflection of hybrid vigor, which is known to result from crossing two highly inbred strains (40). These results clearly demonstrate the importance of comparing the life-spans of strains with identical genetic backgrounds. Moreover, these results, together with those from the transformants, show that even though greatly diminished SOD levels may decrease life-span, additional SOD clearly does not increase the life-span of *Drosophila*.

Our findings do not contradict the free radical theory of aging. Despite the observation that the ratio of SOD levels to specific metabolic rates found in a wide range of animals correlates directly with their longevity (5), *Drosophila* appears to be well protected against the toxic effects of oxygen by its native levels of SOD and catalase.

Another important protective enzyme not considered in this study is mitochondrial Mn SOD. In eukaryotic aerobic organisms, most of the oxygen is metabolized within the mitochondrion. Aging may be the result of accumulated peroxidative damage to mitochondria, resulting in decreased ATP production (41). Since Mn SOD activity represents only 10% of the total SOD activity in *Drosophila* (42), it is possible that Mn SOD rather than Cu–Zn SOD levels are the limiting factor in the aging process.

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