In:MOLECULAR BIOLOGY OF AGING
UCLA Symposia on Molecular and Cellular biology, New series, Volume 123
Editors, M. Clegg & S. O'Brien
Alan R. Liss, Inc. New York, NY (1989)

GENETIC AND MOLECULAR ANALYSIS OF ANTIOXIDANT ENZYMES IN DROSOPHILA MELANOGASTER: A CORRELATION BETWEEN CATALASE ACTIVITY LEVELS, LIFE SPAN, AND SPONTANEOUS MUTATION RATE

William J. Mackay[1,3], William C. Orr[2], and Glenn C. Bewley[1,4]

[1]Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695-7614;
[2]Department of Biological Sciences, Southern Methodist University, Dallas, Texas 75275-0376

ABSTRACT Activated oxygen species have been demonstrated to be the important agents in oxygen toxicity by disrupting the functional and structural integrity of aerobic cells, primarily through damage to DNA, lipids, and proteins. The accumulated effect of oxygen free radical damage is thought to be a contributing factor to the aging process. We are developing genetic and molecular models for antioxidant enzymes that will provide an important source of material to assess the role of free radical damage in biological aging and if antioxidant enzymes play a significant role in minimizing these effects. Catalase and superoxide dismutase are two major antioxidant enzymes involved in scavenging activated oxygen species. We have isolated six acatalasemic mutants and three null SOD mutants in Drosophila melanogaster. We are interested in the role of these enzymes in protecting Drosophila from DNA damage and the relationship between oxyradical induced DNA damage

3) Present Address: Biology Department, Saint Anselm College, Manchester, New Hampshire 03102

4) To whom all correspondence should be addressed (919) 737-2285

and lifespan determination. Both the Sod and catalase genes have been cloned, and to test the prediction that overexpression of antioxidant enzymes can lengthen maximum life span potential of Drosophila, multiple copies of these genes will be integrated into the genome to determine the effect of overexpression on Drosophila life span and mutation rate.

INTRODUCTION

Many studies have provided evidence demonstrating that activated oxygen species are toxic to aerobic cells (1,2). These toxic effects may occur as a result of interactions of these molecules with various cellular components such as DNA (3-6; this report), lipids (7) and proteins (8). It has been hypothesized that oxygen free radical damage is a major factor contributing to a number of aging related diseases and, in general, to the aging process (2,4,9-11). In 1956, Harman (12) first proposed the oxygen free radical theory of aging. This theory states that oxygen free radical reactions, arising largely during the course of normal aerobic metabolism, are responsible for a progressive temporal accumulation of cellular changes that are responsible for the ever-increasing likelihood of disease and death that accompanies advancing age. Our goal here is to develop genetic and molecular models to test several predictions of Harman's theory. What is the magnitude of importance of oxyradical defense scavenging mechanisms during eukaryotic development and senescence? Does free radical damage restrict the maximum life span potential of aerobic organisms? By integrating multiple copies of antioxidant genes into the genome, thus creating in vivo cellular conditions which overexpress the amounts of antioxidant gene products, is it possible to alter or extend the maximum life span potential? Finally, does overexpression of one antioxidant gene product extend, restrict, or have no effect on life span? In other words, must the levels of antioxidant gene products be kept in a stoichiometric balance to achieve, or possibly lengthen an organism's maximum life span potential? A corollary to this prediction is that overexpression of one antioxidant gene-enzyme system without a concommittant increase in the expression of other functionally-related antioxidant genes may actually restrict life span.

Oxygen Free Radical Reactions. The complete tetravalent reduction of molecular oxygen is illustrated in figure 1 where partial oxygen reductants include the superoxide anion, hydrogen peroxide, and the hydroxyl radical (13).

It has been thought that the deleterious effects in aerobic cells associated with endogenously generated prooxidants are due to reactions initiated by the hydroxyl radical, which is produced in a superoxide anion-driven Fenton cycle involving the reduction of hydrogen peroxide in the presence of transition metals (2,13). The hydroxyl radical is highly reactive with most organic molecules found in aerobic cells (14). Although superoxide anion and hydrogen peroxide are less reactive as oxidants, hydrogen peroxide is uncharged and can diffuse to significant distances from its endogenous site of origin before reduction to the hydroxyl radical (2,15).

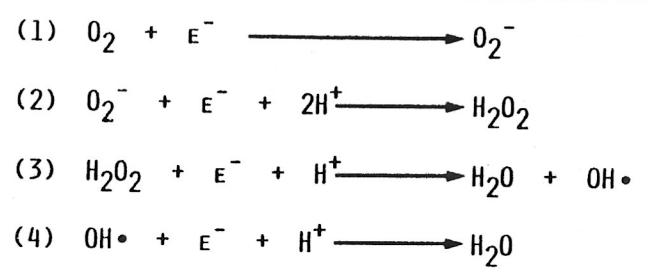

Figure 1. The univalent pathway of oxygen reduction. Equations one through four summarize the sequence of single-electron reductions of dioxygen and the major reduction products that are formed. For a more detailed treatment see Green and Hill (1984).

Enzymatic Antioxidant Defenses. Prokaryotic and eukaryotic aerobic organisms have evolved both enzymatic and non-enzymatic defense mechanisms (i.e. antioxidants) which are thought to provide considerable protection against the effects of oxygen radical-induced genetic and cellular damage (1,2,4,6,9-10). Two major antioxidant enzymes, superoxide dismutase (SOD; superoxide: superoxide oxidoreductase; E.C. 1.15.1.1) and catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase; E.C. 1.11.1.6) function to efficiently remove activated oxygen species. SOD catalyzes the

dismutation of superoxide anion to hydrogen peroxide (16), while catalase catalyzes the breakdown of hydrogen peroxide to water and ground state molecular oxygen (17). By scavenging both superoxide anion and hydrogen peroxide, formation of the highly reactive hydroxyl radical is limited.

We are developing both *in vivo* genetic and molecular systems in order to test the role of antioxidant enzymes in protecting multicellular eukaryotes from oxygen free radical damage during development and senescence. The fruitfly, *Drosophila melanogaster*, is currently the best metazoan to study antioxidant gene-enzyme systems from a combined genetic, biochemical, and molecular approach. The isolation of loss of function mutants for antioxidant enzymes will provide valuable insight into the importance of oxygen free radical defenses on life span parameters, and particularly the relationships between life span, levels of antioxidant enzyme and the rate of DNA damage. A molecular approach will allow the integration of multiple copies of antioxidant genes in transgenic flies in order to determine the effects of overexpression of antioxidant enzymes on life span parameters. In this report we summarize our recent results pertaining to the genetics and molecular biology of the catalase and SOD gene-enzyme systems in *Drosophila melanogaster*.

RESULTS AND DISCUSSION

The Genetics of Catalase in Drosophila melanogaster. The structural gene for Drosophila catalase, Cat^+, has been mapped to position 3-47.0 and has been cytologically localized to the 75D1-75F1 region on the left arm of chromosome three (18,19). Extensive EMS mutagenesis experiments have resulted in the isolation of six acatalasemic mutants (19). Two mutations are amorphic alleles of the locus while the other four are hypomorphic. Subsequent complementation analysis of these mutations have shown that all six mutations are allelic indicating that there is only one functional structural gene for Drosophila catalase within the genome. By crossing each of these six mutants in all pair-wise combinations, heteroallelic combinations were derived which display catalase activity levels ranging from 0% to 50% of normal. Such heteroallelic combinations provide a wide spectrum of

activity levels which can be utilized in studies designed to examine both the phenotypic effects of acatalasemia on life span parameters and to correlate these parameter defects to both germline and somatic mutation frequencies.

The Effect of Acatalasemia on Life Span Parameters in Drosophila. There is a strong positive linear correlation between catalase activity level and life span (figure 2). In fact complete loss of function catalase mutants result in a 75-86% life span reduction. These results are consistent with one prediction of Harman's original theory, that loss of antioxidants should restrict life span in aerobes. Interestingly, even though these low activity strains possess shortened life spans, no apparent defects were observed in early development. See Mackay and Bewley (1989) for a review of the relative viability of acatalasemic mutants. One possible interpretation of these results is that early Drosophila development consists primarily of five distinct morphological stages where, at each stage, most tissues are rapidly generated and then decomposed. Each

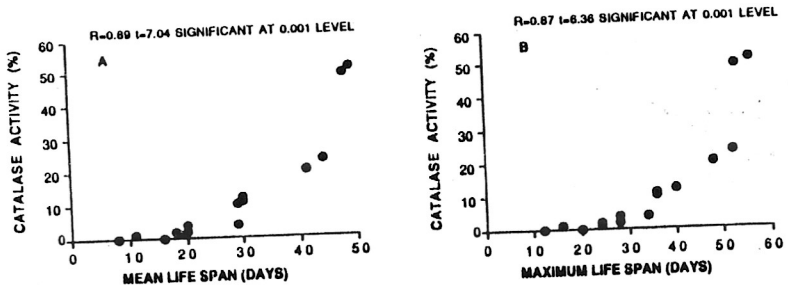

Figure 2. Scatter plots illustrating the relationship between catalase activity and Drosophila life span for the fifteen mutant acatalasemic heteroallelic combinations. The statistical analysis corresponding to each plot is depicted at the top of each panel where **R** = the product-moment correlation coefficient relating enzymatic activity to lifespan and **t** = the student's t test value.

developmental stage is rather short compared to adult lifespan and oxygen initiated cellular damage may not accumulate to toxic levels. Furthermore, most cells of the larval stages are polytenic. Such cells may be less

vulnerable to oxygen initiated cytological damage than dividing diploid cells. Finally, oogenesis may provide a maternal dowry of catalase activity to carry the development of acatalasemic mutant embryos to adulthood. In contrast, adult lifespan is protracted and most adult tissues are postmitotic. Therefore, it is logical to assume that an antioxidant defense mechanism must be kept at maximum level during the lifespan of the adult and that reductions of either non-enzymatic or enzymatic antioxidants could result in an increased accumulation of oxyradical induced cellular damage with subsequent reductions of lifespan.

The relationship between catalase activity and germline mutation frequencies. Much attention has been focused on DNA as the critical target in aging largely because of the central role of DNA in information transfer between generations of somatic cells during the life span of aerobes. There is significant evidence correlating DNA alterations with the aging process in mammals (20). It has also been shown that increased levels of antioxidants can reduce oxidative DNA damage (21). We have employed a genetic approach to address the relationship between the level of catalase activity and, presumably, the rate of oxidative DNA damage. This experiment typically referred to as a Muller-5 (22), allows one to measure the rate of germline mutations, either spontaneous or under mutagenic or carcinogenic environments. In fact, this experimental approach has been widely used to test the mutagenicity and/or carcinogenicity of many agents and chemicals during the last sixty years. The outline of the Muller-5 scheme is illustrated in figure 3. The design of the experiment allows one to isolate independently-derived X-linked recessive lethal mutations in the F2 generation. Adult P1 males are mass-mated to virgin females possessing multiple phenotypic markers, both dominant and recessive, on the sex (X) chromosome. The complete genotype of this strain is B (bar eyes) w^a (white apricot) sc (scute wings). F1 heterozygous virgin females are pair-mated to sibling F1 male progeny. This cross gives rise to 4 progeny classes in the F2 generation. If a recessive lethal mutation has occurred on the X chromosome in the P1 male, this will be noted by the absence of wild type-eyed males in the F2 progeny. In addition, it is also possible to score the presence of a semi-lethal mutation, which we have

arbitrarily defined as an F2 generation that contains less than 5% wild type-eyed males relative to the total F2 progeny.

The rationale of utilizing a Muller-5 to measure germline mutation frequencies in acatalasemics is fairly simple. If catalase plays a role in protecting Drosophila from oxidative DNA damage, then one would expect that the frequency of sex-linked recessive lethal mutations would be higher for a null catalase genetic background relative to wild type. Preliminary results of this experiment comparing the spontaneous mutation

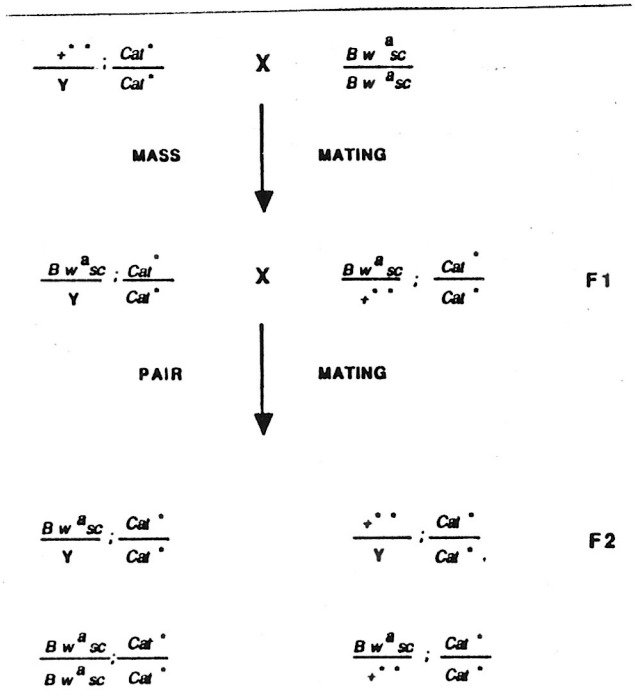

Figure 3. Muller-5 scheme to determine the frequency of sex-linked recessive lethal mutations. The double asterisk illustrates segregation of the X-chromosome through the F2 generation. Absence of male progeny bearing this chromosome among the F2 progeny is scored as a recessive lethal. The single asterisk indicates either a wild type or catalase negative background in the P1 male. See text for a descriptive narrative.

rate for a null catalase mutant, $\underline{Cat}^{n1}/\underline{Cat}^{n4}$, and a leaky mutant, $\underline{Cat}^{n1}/\underline{Cat}^{n2}$ (possessing approximately 4% of wild type catalase activity), to the mutation

frequency of the parental strain having normal levels of catalase activity are depicted in Table 1. The null catalase mutant strain exhibits an 8-fold increase in mutation rate while the catalase hypomorph displays a 6.7-fold increase. In summary, these findings indicate that loss of catalase activity has profound effects on the spontaneous rate of Drosophila germline mutations. We are now repeating these Muller-5 experiments under conditions of oxidative stress to futher define the importance of catalase in scavenging activated oxygen species in vivo.

TABLE 1

FREQUENCY OF SPONTANEOUS SEX-LINKED RECESSIVE LETHAL MUTATIONS

STRAIN	# OF F2 CHROMOSOMES SCREENED	# OF STERILES (%)	# OF LETHAL MUTATIONS AND MUTAGENESIS FREQUENCY[a]	# OF SEMI-LETHAL MUTATIONS AND MUTAGENESIS FREQUENCY[a]	TOTAL MUTAGENESIS FREQUENCY (%)[b]
$\frac{Cat^{+}}{Cat^{+}}$	1062	197 (18%)	4 (0.005 ± 0.002)	1 (0.001 ± 0.000)	0.6
$\frac{Cat^{n1}}{Cat^{n4}}$	635	98 (15%)	26 (0.05 ± 0.009)	1 (0.000 ± 0.000)	5
$\frac{Cat^{n1}}{Cat^{n2}}$	595	100 (17%)	7 (0.014 ± 0.005)	11 (0.02 ± 0.007)	4

a) Number of X-linked recessive lethal and semi-lethal mutations; parenthesis depicts the mutagenesis frequency ± standard error. The mutagenensis frequency represents the number of lethal and/or semi-lethal mutations divided by the number of fertile F2 chromosomes screened.

b) Total mutagenesis frequency represents the number of recessive lethals and semi-lethals divided by the total number of fertile F2 chromosomes screened.

The genetics of Drosophila superoxide dismutases. Although little is known pertaining to the genetics of the mitochondrial manganese SOD, there have been several recent studies pertaining to the genetics of the cytosolic Cu/Zn SOD gene-enzyme system (23,24; J. Phillips, personal communication; G.C. Bewley, unpublished results). The structural gene for Cu/Zn Sod^{+} has been genetically mapped to position 3-34.6 (25), and has been cytologically localized to the 68A5,6-68A8,9 region on the left arm of chromosome three (Bewley and Mackay, unpublished results). Several studies have resulted in the identification of 13 recessive lethal complementation groups within the 68A-C region (23,26), and one semi-lethal mutant which is a hypomorphic allele of the Sod locus (23). Subsequent analysis has revealed that this mutant, $cSOD^{n108}$, is sterile as a homozygote and displays a drastically shortened life span (24). The mutant is also hypersensitive to the superoxide generator, paraquat, and to the transition metal, Cu^{++} (24). Continuing mutagenesis studies have resulted in the isolation of three additional SOD mutants (G.C. Bewley, unpublished results). Unlike $cSOD^{n108}$, these mutations appear to be amorphic and all three display a recessive lethal phenotype. Preliminary complementation analysis has shown that the three amorphic Sod mutations are allelic and that each complement $cSOD^{n108}$ for both enzymatic activity and the recessive lethal phenotype (G.C. Bewley, unpublished results). These SOD mutants now provide the raw material to perform functional studies similar to the experiments that are currently in progress for the acatalasemic mutants. Finally, it is now possible to construct a double Sod Cat mutant to study the effects of an Sod Cat negative genetic background on these same parameters.

Molecular Cloning and Analysis of Antioxidant Genes. In order to test in vivo a second major prediction of Harman's aging theory, namely that an overabundance of antioxidant enzymes can alter, and possibly extend, Drosophila life span potential, the molecular cloning and analysis of antioxidant genes is critical. Considerable work has been accomplished on the molecular dissection of the Sod^{+} locus. Both cDNA and genomic clones have been isolated and sequenced (27), and the DNA sequences derived from these clones are in complete agreement with the known primary amino

acid polypeptide sequence (28). The gene consists of two coding sequences of 22 and 132 codons, respectively, interrupted by a large intron of 725 nucleotides which falls between codons 22 and 23 that is bounded by traditional eukaryotic splice sites. Efforts are presently underway to clone the Drosophila catalase gene. We have purified Drosophila catalase to homogeneity and have raised mono-specific antibodies against this enzyme (29). Using these antibodies, we have screened a Drosophila adult cDNA library constructed in the expression vector lambda-Zap (Stratagene). This library was kindly provided to us by M. Shea and D. Falb (Harvard University). We screened approximately 300,000 recombinant plaques with the catalase antibody and detected 10 positive signals after incubation with an anti-IgG alkaline phosphate conjugate and the appropriate color reaction (30). Of these 10, three were eventually plaque purified and converted to plasmid form via the automatic excision process (Stratagene). Two of these recombinant clones had an insert size of about 1.4 kb while the third had an insert size of 1.2 kb. All three of these clones cross-hybridized, confirming the specific nature of the probe used in this screen. Since the estimated size of the catalase messenger RNA is approximately 2.2 kb (18), these clones do not represent full length catalase cDNAs.

We have two lines of evidence that support the contention the these cDNA clones indeed represent catalase sequence. First, we localized one of our clones by in situ hybridization to the 75D-E region on chromosome 3L (data not shown). This correlates well with the known cytological location of the catalase gene as determined by genetic analysis (18,19). Secondly, we initiated sequencing of these clones and have identified extensive sequence homology between them and previously isolated catalase genes (data to be presented elsewhere). Further proof as to the identity of our cDNA clones should be provided by using them in a differential screen to probe RNAs isolated from acatalasemic mutants, experiments which are now in progress.

CONCLUSION

According to the free radical theory of aging,

the accumulation of oxyradical-induced damage over time is a causal factor in the aging process. At the core of this theory is the notion that anti-oxidant defenses are somehow inadequate. With recent advances in biotechnology, it is now possible to integrate multiple copies of the wild type catalase and Sod genes into the Drosophila genome by P-element mediated transformation. Thus it will be possible to construct transgenic animals with extra copies of antioxidant genes to determine what effect the elevation of antioxidant gene expression might have on life span potential and other aging-related parameters. These studies become particularly intriguing in light of recent results where multiple copies of the Fe-Sod$^+$ gene have been inserted into the bacterium E. coli (31). These transformed cells exhibit an 11-fold increase in total SOD activity, while the level of catalase remains unaffected. Paradoxically, rather than increasing cellular tolerance, these transformed cells are hypersensitive to conditions of oxidative stress. A logical conclusion that stems from these studies is that an effective defense against the toxic effects of free radical damage requires a balance between the levels of antioxidant enzymes which cannot be improved by increases in one class of enzyme alone. This theory is easily testable by integrating multiple copies of the Drosophila catalase and SOD genes into the genome and determining what balance, if any, can actually increase the maximum life span potential of the organism.

ACKNOWLEDGMENTS

We thank Elizabeth Orr, Jillian Hosford, and Patricia B. Ligon for technical assistance. This work was supported by National Institutes of Health Research Grant No. AG01739 to GCB and by National Research Award No. AG05380 to WJM. This is paper _____ of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

REFERENCES

1. Fridovich I (1977). Oxygen is toxic! Bioscience 27:462.

2. Halliwell B, Gutteridge GMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1.
3. Saul RL, Gee P, Ames BN (1987). Free radicals,DNA damage, and aging. In Warner HR, Butler RN, Sprott LR, Schneider EL (eds): "Modern Biol. Theories of Aging," New York: Raven Press, p 113.
4. Cerutti PA, (1987) The role of DNA damage and its repair in aging; future directions of research. In Warner HR, Butler RN, Sprott LR, Schneider EL (eds): "Modern Biol. Theories of Aging," New York: Raven Press, p 199.
5. Adelman R, Saul RL, Ames BN (1988). Oxidative damage to DNA: Relation to species metabolic rate and life span. PNAS USA 85:2706.
6. Imlay JA, Linn S (1988). DNA damage and oxygen radical toxicity. Science 240:1302.
7. Porter NA (1984). Chemistry of lipid peroxidation. In Packer, L (ed): "Methods of Enzymol 105, New York: Academic Press, p 273.
8. Davies KJA (1987). Protein damage and degradation by oxygen radicals I. General aspects. J Biol Chem 262:9895.
9. Ames, BN (1983). Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases. Science 221:1256.
10. Cerutti, PA (1985). Prooxidant states and tumor promotion. Science 227:375.
11. Pryor, WA (1987). The free-radical theory of aging revisited: A critique and a suggested disease-specific theory. In Warner HR, Butler RN, Sprott LR, Schneider EL (eds): "Modern Biol. Theories of Aging," New York: Raven Press, p 89.
12. Harmon D (1956). A theory based on free radical and radiation chemistry. J Geront 11:298.
13. Green MG, Hill HAO (1984). Chemistry of dioxygen. In Packer, L (ed): "Methods of Enzymol 105, New York: Academic Press, p 3.
14. Czopski G (1984). Reaction of $^{\cdot}OH$. In Packer, L (ed): "Methods of Enzymol 105, New York: Academic Press, p 209
15. Brunori M, Rotillio G (1984). Biochemistry of oxygen radical species. In Packer, L (ed): "Methods of Enzymol 105, New York: Academic Press, p 22.
16. Fridovich I (1986). Superoxide dismutases. Adv Enzymol 58:61.

17. Aebi HE (1984). Catalase in vitro. In Packer, L (ed): "Methods of Enzymol 105, New York: Academic Press, p 121.
18. Bewley GC, Mackay WJ, Cook JL (1986). Temporal variation for the expression of catalase in Drosophila melanogaster: correlations between the rates of enzyme synthesis and levels of translatable catalase-messenger RNA. Genetics 113:919.
19. Mackay WJ, Bewley GC (1989) The genetics of catalase in Drosophila melanogaster: Isolation and characterization of acatalasemic mutants. Genetics 122: in press.
20. Gensler HL, Hall JD, Bernstein H (1987). The DNA damage hypothesis of aging: Importance of oxidative damage. Rev Biol Res Aging 3:451.
21. Lawson T, Stohs S (1985). Changes in endogenous DNA damage in aging mice in response to butylated hydroxyanisole and oltipraz. Mech Ageing Dev 30:179.
22. Spencer WP, Stern C (1948). Experiments to test the validity of the linear R-dose/mutation frequency relation in Drosophila at low dosage. Genetics 33:43.
23. Campbell SD, Hilliker AJ, Phillips JP (1986). Cytogenetic analysis of the cSOD microregion in Drosophila melanogaster. Genetics 112:205.
24. Phillips JP, Campbell SD, Michaud D, Charbonneau M, Hilliker AJ (1989). A null mutation of cSOD in Drosophila confers hypersensitivity to paraquat and reduced longevity. PNAS USA 86:2761.
25. Schott D, Olson MC, Finnerty V (1986) Molybdenum hydroxylases in Drosophila III. Characterization of the low xanthine dehydrogenase gene. Biochem Genet 24:509.
26. Crosby MA, Meyerowitz EM (1986). Lethal mutations flanking the 68C glue gene cluster on chromosome 3 of Drosophila melanogaster. Genetics 112:785.
27. Seto NOL, Hayashi S, Tener GM (1989). Cloning, sequence analysis and chromosomal localization of the Cu/Zn superoxide dismutase gene of Drosophila melanogaster. Gene 75:85.
28. Lee YM, Freidman DJ, Ayala FJ (1985). Complete amino acid sequence of copper-zinc superoxide dismutase from Drosophila melanogaster. Arch Biochem Biophys 241:577.

29. Nahmias JA, Bewley GC (1984). Characterization of catalase purified from Drosophila melanogaster by hydrophobic interaction chromatography. Comp. Biochem. Physiol. [B] 77:355.
30. Mierendorf RC, Perry C, Young RA (1987). Gene isolation by screening lambda gtll libraries with antibodies. In Packer, L (ed): "Methods of Enzymol 152,New York: Academic Press, p 458.
31. Scott MD, Meshnick SR, Eaton JW (1987). Superoxide dismutase-rich bacteria: Paradoxical increase in oxidant toxicity. J Biol Chem 265:3640.