The Genetics of Catalase in *Drosophila melanogaster*: Isolation and Characterization of Acatalasemic Mutants

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ABSTRACT

Activated oxygen species have been demonstrated to be the important agents in oxygen toxicity by disrupting the structural and functional integrity of cells through lipid peroxidation events, DNA damage and protein inactivation. The biological consequences of free radical damage have long been hypothesized to be a causal agent in many aging-related diseases. Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.15.1.1) is one of several enzymes involved in the scavenging of oxygen free radicals and free radical derivatives. The structural gene for catalase in *Drosophila melanogaster* has been localized to region 75D1-76A on chromosome *3L* by dosage responses to segmental aneuploidy. This study reports the isolation of a stable deficiency, *Df(3L)Cat*^{DH104}*(75C1-2;75F1)*, that uncovers the catalase locus and the subsequent isolation of six acatalasemic mutants. All catalase mutants are viable under standard culture conditions and recessive lethal mutations within the 75C1-F1 interval have been shown not to affect catalase activity. Two catalase mutations are amorphic while four are hypomorphic alleles of the *Cat*$^+$ locus. The lack of intergenic complementation between the six catalase mutations strongly suggests that there is only one functional gene in Drosophila. One acatalesemic mutation was mapped to position 3-47.0 which resides within the catalase dosage sensitive region. While complete loss of catalase activity confers a severe viability effect, residual levels are sufficient to restore viability to wild type levels. These results suggest a threshold effect for viability and offer an explanation for the general lack of phenotypic effects associated with the known mammalian acatalasemics.

THE complete reduction of molecular oxygen to water proceeds through the sequential acceptance of four electrons (reviewed by GREEN and HILL 1984). As a by-product of this pathway toxic single electron reduction products, namely the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH·), result through a variety of metabolic processes or when an organism is exposed to a number of environmental agents (FRIDOVICH 1977; AMES 1983; HALLIWELL and GUTTERIDGE 1984; CERUTTI 1985). In general, the accumulated affect of oxygen free radical damage is thought to be a contributing factor to aging, carcinogenesis and tumor promotion, and a number of aging-related disorders (AMES 1983; CERUTTI 1985, 1987; HALLIWELL and GUTTERIDGE 1984; HARMAN 1956, 1984; SAUL, GEE and AMES 1987).

Aerobic organisms have evolved both nonenzymatic and enzymatic defense mechanisms to remove activated oxygen species and to provide protection against the effects of oxygen radical induced cellular and genetic damage (FRIDOVICH 1977; AMES 1983; HALLIWELL and GUTTERIDGE 1984; CERUTTI 1985, 1987; IMLAY and LINN 1988). Two major antioxidant enzymes, superoxide dismutase (SOD; superoxide: superoxide oxidoreductase; EC 1.15.1.1) and catalase (CAT; H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6), are functionally coupled to efficiently remove activated oxygen species. SOD catalyzes the dismutation of superoxide anion to H_2O_2 and oxygen (FRIDOVICH 1986), while catalase catalyzes the breakdown of H_2O_2 to water and molecular oxygen (AEBI 1984). By scavenging the superoxide anion and H_2O_2, formation of the highly reactive hydroxyl radical is limited.

The development of a genetic model for catalase and SOD is necessary to directly study the importance of each enzyme in protecting aerobic cells from oxygen free radical damage. Acatalasemia has been reported in humans and in the mouse (TAKAHARA 1968; AEBI *et al.* 1968; FEINSTEIN *et al.* 1966), but subsequent studies have revealed that these mutants actually express significant levels of catalase activity in solid tissues and should more correctly be termed hypocatalasemic (AEBI and WYSS 1978; SHAFFER, SUTTON and BEWLEY 1987). Recent studies have reported the isolation of null catalase mutants in the bacterium *E. coli* and the yeast *S. cerevisiae* (LOEWAN *et al.* 1985; COHEN *et al.* 1985), and while these mutant cells are viable under standard culture conditions, they are hypersensitive to environments of oxidative stress.

The ultimate goal of this project is to assess the importance of catalase in protecting DNA against the damaging effects of oxygen free radicals and the

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relationship of DNA damage to aging and senescence in Drosophila. The structural gene for Drosophila catalase, *Cat*$^+$, has been mapped to the cytogenetic interval 75D1-76A on the left arm of chromosome *3* (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984). The enzyme has been purified to homogeneity, is tetrameric with a subunit molecular weight of 58,000 and catalase-monospecific antibodies have been raised (NAHMIAS and BEWLEY 1984). Two distinct peaks of catalase activity are observed during Drosophila development with the first peak occurring in late third instar larvae just prior to puparium formation and the second and larger of the two peaks occurring during metamorphosis (BEWLEY, NAHMIAS and COOK 1983). Upon emergence of the adult, catalase activity reaches a steady state level. A number of variants to this basic program have been isolated that affect the quantitative, temporal, and tissue specific expression of the structural gene, and two temporal variants have been mapped to position 3-47.0 on the left arm of chromosome *3* where the catalase structural gene resides (BEWLEY and LAURIE-AHLBERG 1984; BEWLEY, MACKAY and COOK 1986). In this study we report the isolation of the first stable deficiency which uncovers the catalase region in Drosophila and the isolation and genetic characterization of the first null catalase mutants in any multicellular eukaryotic organism.

MATERIALS AND METHODS

Genetic stocks and treatment: Drosophila cultures were maintained at 25° in uncrowded shell vials or half-pint bottles on standard cornmeal-molasses-yeast-agar medium containing propionic acid and Tegosept-M as mold inhibitors.

The multiply marked third chromosome strains used in the mutagenesis experiments, *ri sbd e*^{2} and *cp in ri p*^{p}, were obtained from the Bowling Green National Drosophila Stock Center. These strains were isogenized for the third chromosome and placed into a highly inbred background line (Ho-R) for chromosomes *1* and *2*. The multiply marked third chromosome strains used for mapping studies, *ru cu ca* and *ru Pri ca*, have been previously described (BEWLEY, MACKAY and COOK 1986). The *In(3LR)TM3* balancer chromosomes contain the recessive markers *y*$^+$ *ri p*^{p} *sep bx*^{34e} *e*^{s} in addition to either the dominant markers *Sb Ser* (*TM3, Sb Ser*) or *Ser* (*TM3, Ser*). All genetic symbols are described in LINDSLEY and GRELL (1968).

The following deficiency chromosomes were utilized: *Df(3L)W*^{r4}*(75B8-11;75C5-7)*, *ru h sbd*^{2} *ro ca* was provided by WILLIAM SEAGRAVES; *Df(3L)lxd*^{9}*(68A4;68B4-C1)*, *v; cur* was obtained from VICTORIA FINNERTY (SCHOTT, OLSON and FINNERTY 1986); *Df(3L)VW3(76A3;76B2)* and *Df(3L)in*^{61j1}*(76F;77D)* were provided by the UMEA Drosophila Stock Center and have been previously described (ASBURNER *et al.* 1981; ARAJARVI and HANNA-ALAVA 1969); *Df(3L) Cat*^{DH104}*(75C1-2;75F1)*, *ri sbd e*^{2} was isolated during the course of this study.

Isolation of deficiencies within the 75 region on 3L: *Df(3L)W*^{r4}, a γ-ray-induced deletion of 75B8-75C7, was used to screen for additional recessive lethal mutations within this interval. Adult males aged 0–48 hr bearing the following third chromosome markers *ri sbd e*^{2} were exposed to 4000 rad of γ-rays and mated to *Df(3L)W*^{r4}*/TM3, Ser* virgin females. F_1 **ri sbd e*^{2}*/TM3, Ser* males were individually mated to three *Df(3L)W*^{r4}*/TM3, Ser* virgin females. The absence of **ri sbd e*^{2}*/Df(3L)W*^{r4} F_2 progeny indicated the presence of a recessive lethal mutation on the *ri sbd e*^{2} chromosome when in combination with *Df(3L)W*^{r4} and this mutation was subsequently recovered by collecting sibling **ri sbd e*^{2}*/TM3, Ser* F_2 progeny. Chromosomes bearing lethal mutations were subsequently assayed as heterozygotes for catalase activity.

The polytene chromosomes of **ri sbd e*^{2} third instar larvae bearing recessive lethal mutations that exerted a gene-dosage effect for catalase activity were examined for cytological aberrations within the 75 region. Larvae were grown at 22° on CARPENTER's (1950) media under uncrowded conditions. Late third instar larvae were dissected in 45% acetic acid and the salivary glands were fixed for several minutes in lactic-acetic orcein for standard chromosome squashes.

Isolation of acatalasemic mutations: A standard Drosophila F_2 mutagenesis screen was conducted to isolate both amorphic and hypomorphic catalase mutations. Male *cp in ri p*^{p} adults aged 0–48 hr were exposed to the alkylating agent ethyl methanesulfoniate (EMS) according to the method of LEWIS and BACHER (1968) and subsequently mated to *Ubx/TM3, Sb Ser* virgin females. F_1 **cp in ri p*^{p}*/TM3, Sb Ser* males were individually mated to three *Df(3L)Cat*^{DH104}*/TM3, Ser* virgin females. The absence of **cp in ri p*^{p}*/Df(3L)Cat*^{DH104} progeny indicated the presence of a recessive lethal mutation on the *cp in ri p*^{p} chromosome when in combination with *Df(3L)Cat*^{DH104}, and this mutation was recovered by collecting sibling **cp in ri p*^{p}*/TM3, Ser* F_2 sibling individuals.

A very sensitive and rapid qualitative assay which we call the "fizz" test was developed to screen for putative hemizygous viable **cp in ri p*^{p}*/Df(3L)Cat*^{DH104} EMS-induced catalase mutants. This assay is based on the enzymatically catalyzed decomposition of hydrogen peroxide to molecular oxygen and the evolution of oxygen bubbles in solution when a concentrated hydrogen peroxide solution is used as substrate. Single adults were homogenized in a microtiter well containing 25 μl of 0.1 M Tris-PO_4 at pH 7.0, and 0.1% Triton X-100. Twenty-five microliters of commercial 30% hydrogen peroxide was subsequently added and the presence of catalase activity in each fly was noted by the rapid appearance of oxygen bubbles. As a control, adult flies made artificially acatalasemic by the dietary administration of the non-competitive inhibitor 3-amino-1,2,4-triazole (AT) showed very little or no bubbling in this qualitative assay.

Developmental staging: Synchronous cultures were established as previously described (BEWLEY 1981), using morphological criteria for pupae as described by BAINBRIDGE and BOWNES (1981).

Enzyme preparation and assay: The preparation of crude enzyme extracts from each developmental stage has been previously described (BEWLEY, NAHMIAS and COOK 1983).

Catalase activity was quantitated by the spectrophotometric method of BEERS and SIZER (1952), and has been previously described for Drosophila (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984). The disappearance of H_2O_2 was monitored at 240 nm, where one unit of activity is defined as 1 μl of H_2O_2 decomposed per minute, based on a molar absorptivity for H_2O_2 of 62.4 (NELSON and KIESOW 1972).

Superoxide dismutase (SOD) activity was determined by

the cytochrome *c* reduction method of McCORD and FRIDOVICH (1969). Ten newly eclosed adults were homogenized in 0.2 ml 0.05 M KPO_4, 0.1 mM EDTA, pH 7.8. One unit of SOD activity is defined by a 50% reduction of cytochrome *c* per minute.

Protein content was determined by the method of LOWRY *et al.* (1951).

Western blots: Western-blot analysis was conducted as previously described (TOBIN, STAEHLIN and GORDEN 1979; BEWLEY, MACKAY and COOK 1986). Total protein from crude extracts of adult flies was separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), and electroblotted onto nitrocellulose. The filter was overlaid first with purified anticatalase IGg and second with goat-anti rabbit IGg conjugated with horseradish peroxidase and developed according to instructions provided by the supplier (Bio-Rad).

Relative viability of acatalasemic mutants: Relative viability was determined by crossing in both directions all pairwise catalase heterozygous mutant combinations (*i.e.*, $Cat^{mutation\ A}/TM3, Sb\ Ser \times Cat^{mutation\ B}/TM3, Sb\ Ser$. Since the TM3 balancer is lethal as a homozygote, the expected Mendelian frequency of heteroallelic catalase mutant combinations should represent 33% of the total F_1 progeny. The parental wild type chromosome *cp in* Cat^+ *ri* p^p segregated in this cross at expected Mendelian frequencies (data not shown) and these values have been normalized to 100%. For each pair-mating approximately 300–500 F_1 progeny were scored.

Dietary administration of hydrogen peroxide: Fifty males or female adults aged less than 24 hr were placed on a 1% agar medium overlaid with Kimwipes containing 2% sucrose and either 0.01%, 0.05%, 0.10% or 1% hydrogen peroxide. Each population was transferred daily onto fresh agar medium containing hydrogen peroxide and the daily mortality rate for each culture was calculated up to 120 hr posteclosion. Control experiments consisted of wild-type and mutant strains exposed to sucrose solutions not containing hydrogen peroxide. For each concentration of hydrogen peroxide three male and three virgin female cultures were observed for each strain.

RESULTS

Isolation of a stable deficiency uncovering the catalase locus: A schematic representation of the left arm of chromosome 3 where the catalase structural gene resides is illustrated in Figure 1. The top line depicts the limits of the catalase dosage sensitive region from 75D1 to 76A based on the autosomal breakpoints of the reciprocal translocation stocks *T(Y;3)L131* and *T(Y;3)B132* (LINDSLEY *et al.* 1972; LINDSLEY and ZIMM 1986), and this is the only dosage sensitive region for catalase within the Drosophila genome (LUBINSKY and BEWLEY 1979). Above the chromosome are the map positions and approximate cytological locations of several visible phenotypic markers while below the chromosome are the cytological breakpoints of known deficiencies within this region. Two deficiencies flank the proximal side of the catalase dosage sensitive region, $Df(3L)in^{61j1}$ and *Df(3L)VW3*, while one deficiency flanks the distal side of the dosage sensitive region, $Df(3L)W^{r4}$. Heterozygotes for these existing deficiencies possess normal levels of catalase activity (data not shown). Thus, the

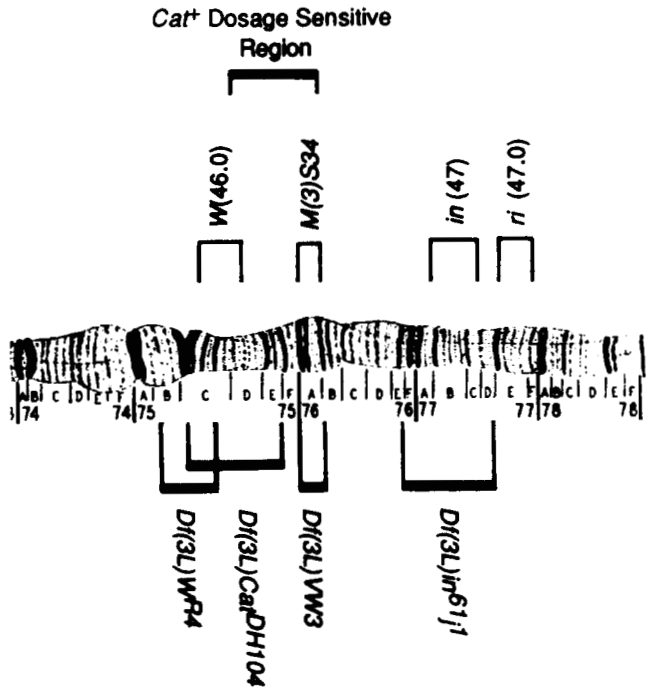

FIGURE 1.—Relationship of genetic variants, including both visible markers and chromosomal deficiencies, that flank and/or include the catalase dosage sensitive region on the left arm of chromosome three. The top line depicts the limits of the dosage sensitive region from 75D1 to 76A3 defined by screening the entire Drosophila genome by segmental aneuploidy (LUBINSKY and BEWLEY 1979).

cytological boundaries of the catalase dosage sensitive region extend from 75D1 to 76A2, based on the autosomal breakpoint of *T(Y;3)L131* (LINDSLEY *et al.* 1972), and the distal breakpoint of *Df(3L)VW3* (ASHBURNER *et al.* 1981).

Since the catalase dosage sensitive region was devoid of stable deficiencies, we began our genetic analysis of the Cat^+ locus by isolating deficiencies which would uncover the 75D1-76A2 region. A standard Drosophila F_2 screen was employed to detect and recover γ-ray-induced recessive lethal mutations when combined with $Df(3L)W^{r4}$ (see MATERIAL AND METHODS). A recessive lethal mutation that exhibited a gene-dosage effect for catalase activity as a heterozygote would be a strong candidate for a deficiency with a distal breakpoint in the interval uncovered by $Df(3L)W^{r4}$ and extending proximal into the catalase dosage sensitive region. We scored 8236 chromosomes and recovered 37 recessive lethal mutations. These mutations were assayed as heterozygotes in combination with the parental Cat^+ *ri sbd* e^2 chromosome for catalase activity to determine if any exerted a gene-dosage effect and 36 exhibited normal levels of catalase activity (data not shown). However, one mutation, *DH104*, exhibited a gene-dosage effect for catalase activity as a heterozygote (Table 1, line 2). Cytologically, *DH104* was defined as a deficiency with a distal breakpoint within the 75C1,2 doublet and a

TABLE 1

Determination of catalase and superoxide dismutase activities in mutants induced at the catalase locus

Parental genotype[a]	Catalase activity	Superoxide dismutase activity
cp in ri p^p/ri sbd e^2	152 ± 3.0	7.2 ± 0.4
cp in ri p^p/Df(3L)Cat^{DH104}	64 ± 5.0	7.2 ± 0.4
cp in ri p^p/Df(3L)lxd^9	149 ± 6.0	3.7 ± 0.3
Cat^{n1}/Df(3L)Cat^{DH104}	0 ± 0.0	7.3 ± 0.2
Cat^{n2}/Df(3L)Cat^{DH104}	7.7 ± 2.8	7.4 ± 0.2
Cat^{n3}/Df(3L)Cat^{DH104}	2.9 ± 0.8	7.2 ± 0.3
Cat^{n4}/Df(3L)Cat^{DH104}	0 ± 0.0	7.2 ± 0.2
Cat^{n5}/Df(3L)Cat^{DH104}	3 ± 1.5	7.4 ± 0.1
Cat^{n6}/Df(3L)Cat^{DH104}	6 ± 2.5	7.4 ± 0.2

All enzyme activities are expressed as units per mg protein ± 1 SD. Activities were determined by triplicate assays from each of three separate extracts.

[a] Catalase mutations Cat^{n1-n6} were independently induced on a chromosome containing the markers *cp in ri p^p*. Df(3L)Cat^{DH104} was induced on a chromosome containing the markers *ri sbd e^2*.

proximal breakpoint at 75F1 (Figure 2) and was subsequently renamed Df(3L)Cat^{DH104}. The results of these studies further delimit the cytological boundaries of the catalase dosage sensitive region from 75D1 to 75F1 based on the autosomal breakpoint of *T(Y;3) L131* and the proximal breakpoint of Df(3L)Cat^{DH104} (Figure 1; LUBINSKY and BEWLEY 1979).

Isolation of catalase mutations: Since there are no reports pertaining to the isolation of null mutants for catalase in multicellular eukaryotes, the phenotype of complete loss of function alleles at the Cat^+ locus in Drosophila could not be predicted *a priori*. Therefore, the F_2 screen outlined in Figure 3 was designed to isolate both recessive lethal mutations within the 75C1-F1 interval in addition to viable acatalasemic mutants. A total of 4512 F_2 chromosomes were scored and 35 recessive lethal mutations were recovered in addition to six viable **cp in ri p^p/Df(3L)Cat^{DH104}* individuals which tested negative for catalase activity under conditions of the "fizz" test (Figure 4). The 35 recessive lethal mutations were subsequently assayed spectrophotometrically as heterozygotes when combined with the parental Cat^+ chromosome. These assays revealed that all 35 recessive lethal heterozygotes had normal levels of catalase activity relative to the parental wild-type controls (data not shown). Of the six viable **cp in ri p^p/Df(3L)Cat^{DH104}* strains that tested negative by the "fizz" test, **cp in Cat^{n1} ri p^p/Df(3L)Cat^{DH104}* (Cat^{n1}) and **cp in Cat^{n4} ri p^p/Df(3L)Cat^{DH104}* (Cat^{n4}) had no detectable levels of catalase activity, while the other four Cat^{n2}, Cat^{n3}, Cat^{n5} and Cat^{n6} had very low levels ranging from 3 to 10% of normal (Table 1).

The level of catalase cross-reacting material (CRM) in the six acatalasemic mutants was determined by Western-blot analysis. The two null catalase activity strains, Cat^{n1} and Cat^{n4}, have no detectable amounts of catalase-specific cross-reacting material (CRM^-) while the other four strains, Cat^{n2}, Cat^{n3}, Cat^{n5} and Cat^{n6}, have low but detectable amounts of CRM that, for the most part, coincide with the level of enzymatic activity in each mutant strain (Figure 5).

Each of the six acatalasemic strains were subsequently assayed to determine what effect the loss of catalase had on the functionally-coupled enzyme SOD. A deficiency uncovering the SOD locus was obtained from VICTORIA FINNERTY (SCHOTT, OLSON and FINNERTY 1986) and as a control, Df(3L)lxd^9/*cp in* Cat^+ *ri* p^p heterozygotes were assayed for SOD activity. These individuals exhibit SOD activity approximately 50% that of wild type while catalase activity is unaffected (line 3, Table 1). Subsequent assays for SOD in all six mutant catalase strains displayed wild-type levels of SOD activity (Table 1).

Developmental profile of catalase activity for Cat^{n1} and Cat^{n2}: The level of catalase activity throughout Drosophila development was determined for the parental *cp in* Cat^+ *ri* p^p stock and for the two homozygous acatalasemic strains, Cat^{n1} and Cat^{n2} (Figure 6). Two distinct peaks of activity for the Cat^+ strain are observed with the first peak occurring in late third instar larvae at approximately 96–100 hr after egg deposition and just before puparium formation. The second and larger of the two peaks occurs during metamorphosis at approximately 190 hr after egg deposition. Catalase activity then declines rapidly and reaches a steady state level in young adults that is approximately one-third of the previous maximum value. This developmental pattern for catalase expression has been observed in many Drosophila strains independently isolated from natural populations (BEWLEY, NAHMIAS and COOK 1983; BEWLEY and LAURIE-AHLBERG 1984). In contrast, both Cat^{n1} and Cat^{n2} do not display the parental wild-type pattern of catalase expression. Cat^{n2} is a hypomorphic allele of the locus exhibiting less than 10% activity throughout development while Cat^{n1} is completely devoid of catalase activity and can therefore be considered an amorphic allele of the Cat^+ locus.

Recombinational mapping of an acatalasemic mutation: The map position of the EMS-induced mutation Cat^{n1} was determined by utilizing the multiply marked third chromosomes *ru cu ca* and *ru Pri ca* as described previously (BEWLEY, MACKAY and COOK 1986). From a total of 1986 F_2 chromosomes 20 recombinational events were isolated between the visible markers *st* (3–44.0) and *cu* (3–50.0). Cat^{n1} recombinant chromosomes bearing either *st* or *cu* would exhibit, when in combination with a Cat^+ chromosome, approximately 50% catalase activity relative to wild type. The Cat^+/Cat^- recombinant individuals representing the 20 independent recombination events were assayed for catalase activity and there

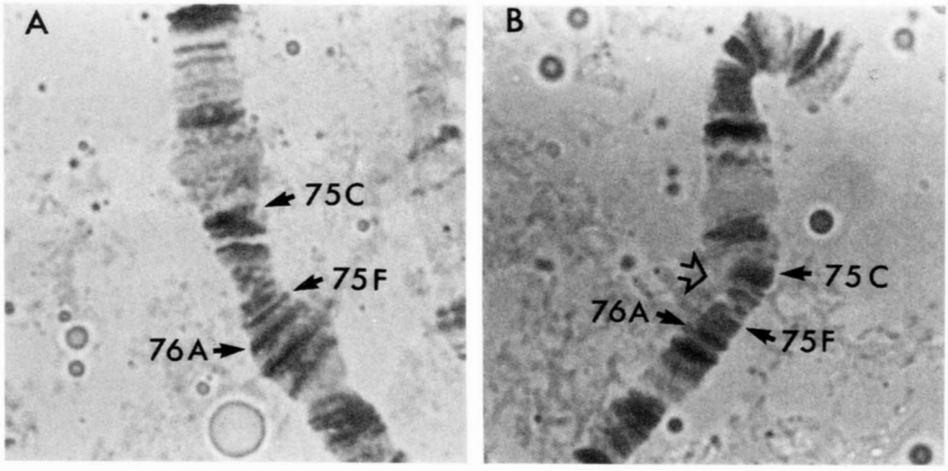

FIGURE 2.—Polytene chromosomes depicting the 75 region on *3L*. Panel A shows a standard chromosome from a third instar larva whose genotype is Cat^{+} *ri sbd* e^{2}. Panel B illustrates a chromosome from a third instar larva whose genotype is $Df(3L)Cat^{DH104}$. Cat^{-} *ri sbd* e^{2}/Cat^{+} *ri sbd* e^{2}.

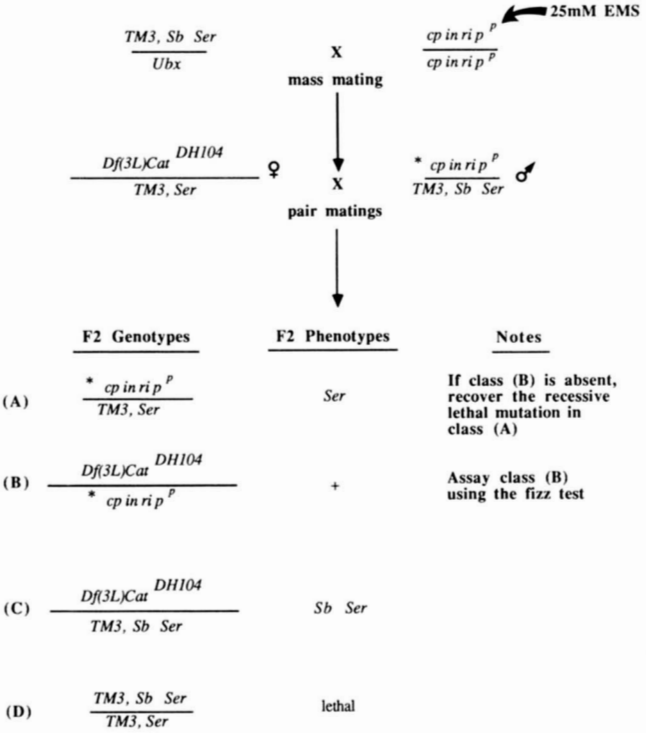

FIGURE 3.—Screen for EMS-induced mutations within the 75C1-F1 interval uncovered by $Df(3L)Cat^{DH104}$. All stocks used in this screen are lethal free and are co-isogenic for the *X* and second chromosomes (i_1/i_1; i_2/i_2; $+_3/+_3$) where *i* represents a chromosome from the original isogenic stock, Ho-R, and + represents the multiply marked chromosome *cp in ri* p^{p}.

existed an equal segregation frequency of the Cat^{n1} mutation relative to the visible flanking markers *st* and *cu* (data not shown). These results place the map position of the Cat^{n1} allele at 3–47.0 with a 95% confidence interval of ± 0.4.

Sensitivity of catalase mutants to the presence of H_2O_2: Figure 7 illustrates the mortality rate of adult flies fed on differing concentrations of H_2O_2. Panels A and B illustrate that the control strains *cp in* Cat^{+} *ri* p^{p}/Cat^{+} *ri sbd* e^{2} and $Df(3L)Cat^{DH104}/cp$ *in* Cat^{+} *ri* p^{p} are resistant to H_2O_2 concentrations of 0.1%, which

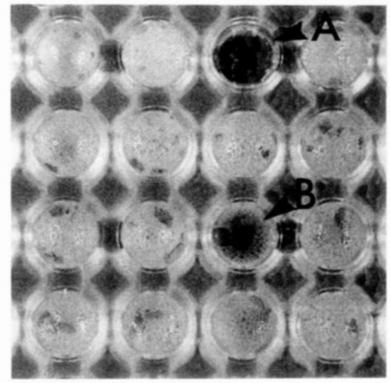

FIGURE 4.—The "fizz" test used to rapidly screen chromosomes for acatalasemic mutations. A single fly from each culture is squashed in 25 μl of buffer and then 25 μl of a 30% commercial solution of hydrogen peroxide is added. The presence of catalase activity is detected immediately by the evolution of oxygen bubbles due to the rapid enzymatic decomposition of hydrogen peroxide. Well A contains a fly of the genotype $Df(3L)Cat^{DH104}/Cat^{n1}$ isolated from the screen in Figure 3. Well B contains a fly made acatalasemic using the noncompetitive inhibitor 3-amino-1,2,4-triazole. Note the few bubbles generated with approximately 5% wild-type activity.

indicates that a 50% reduction of catalase activity has little or no effect on H_2O_2 sensitivity within this concentration range. In addition, the threshold for H_2O_2 tolerance in these same flies is close to 1% since this concentration will eliminate a population within 3 to 4 days. In contrast, both the amorphic mutant $Cat^{n1}/Df(3L)Cat^{DH104}$ and the hypomorphic mutant $Cat^{n2}/Df(3L)Cat^{DH104}$ exhibit a hypersensitive phenotype to H_2O_2 under these same conditions (panels C and D). However Cat^{n2}, which has 5% of the wild type level of catalase activity, exhibits a two-fold increase in resistance over what is observed for Cat^{n1}. These observations suggest that catalase levels of only a few percent confer significant protection against the lethal effects of H_2O_2. It is also worth noting that the control strains in panels A and B mimic the hypersensitive phenotype of Cat^{n1} and Cat^{n2} when treated with 5 mM AT, a noncompetitive inhibitor of catalase (data not shown).

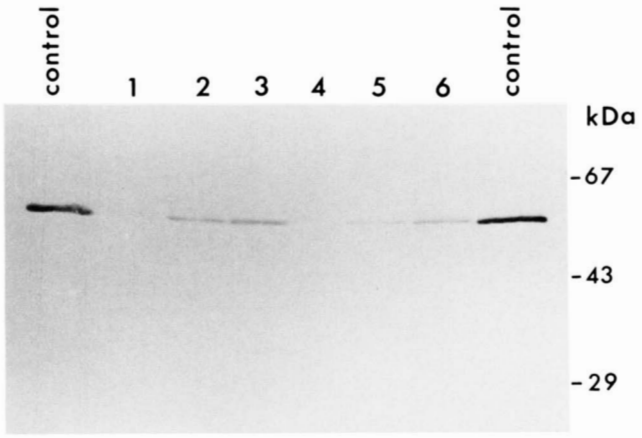

FIGURE 5.—Western blot of total Drosophila protein separated on 12.5% SDS-PAGE and electroblotted onto nitrocellulose. Samples were homogenized at a concentration of one individual per 50 μl and 30 μl of each extract was applied to the gel. The *Df(3L)Cat*^{DH104}*/cp in ri p*^{p} heterozygous strain was used as a control. Lanes 1 through 6 represent the six catalase mutants *Cat*^{n1} through *Cat*^{n6}, respectively, as hemizygotes in combination with *Df(3L)Cat*^{DH104}.

Complementation analysis of catalase mutants: Figure 8 illustrates a complementation matrix of the six catalase mutants for adult catalase activity. The top row shows the level of activity for each of the six acatalasemic heterozygotes relative to the parental wild type strain. Two amorphic alleles, *Cat*^{n1} and *Cat*^{n4}, and two hypomorphic catalase alleles, *Cat*^{n3} and *Cat*^{n5}, display a strict gene-dosage effect for catalase activity as heterozygotes where the level of enzymatic activity is approximately one-half of normal (row 1, columns 2, 4, 5 and 6). However, one acatalesemic mutation, *Cat*^{n6}, displays positive complementation for adult catalase activity when in combination with a *Cat*$^{+}$ chromosome. Since the *Cat*^{n6} allele exhibits approximately 5% of normal adult catalase activity when hemizygous in combination with *Df(3L)Cat*^{DH104} (Table 1), heterozygotes would be expected to possess approximately 55% of wild-type catalase levels. Nevertheless, *Cat*^{n6} heterozygotes display nearly wild-type levels of activity (row 1, column 7). Adult catalase activities were also determined for the fifteen acatalasemic heteroallelic combinations. Eleven heteroallelic combinations exhibit less than 12% catalase activity relative to wild type while two have activity within the 20% range. These activity levels can be explained by additive effects for activity for each chromosome. However, two combinations display positive complementation for enzymatic activity to 50% of normal. Interestingly the leaky CRM^{+} mutants, *Cat*^{n2}, and *Cat*^{n3}, exhibit positive complementation only when combined with the *Cat*^{n6} mutation and not in combination with any other mutans. No heteroallelic combination gave rise to complete restoration of adult catalase activity.

Relative viability of catalase mutants: Scatter plots

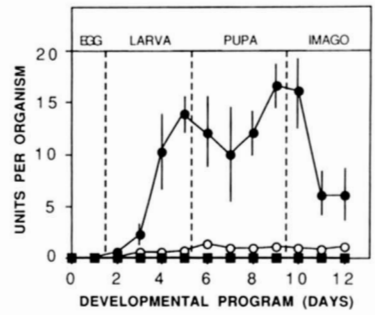

FIGURE 6.—Developmental expression of catalase activity. Each point is the mean of three separate determinations ± 1 SD where (●) represents the *cp in ri p*^{p} parental strain and (■) and (○) represent homozygous *Cat*^{n1} and *Cat*^{n2} strains, respectively. Enzymatic activity is expressed as units per organism.

illustrating the relationship between relative viability of Drosophila as a function of adult catalase activity for all 15 heteroallelic combinations are presented in Figure 9A, where the activity level of each mutant combination ranges from 0% to 50% of normal (Figure 8). Statistical analyses revealed that there is no apparent correlation between relative viability and adult catalase activity at a P value greater than 0.10. However, a different level of significance is obtained with the subset of heteroallelic combinations exhibiting less than 3% of normal adult activity (Figure 9B). This plot shows a highly significant correlation between catalase activity and relative viability at a P value less than 0.01. Such observations suggest a threshold level of catalase activity above which is sufficient to promote Drosophila development with no discernible phenotypic or viability effects.

DISCUSSION

This study reports the isolation of a stable deficiency uncovering the 75C1,2-75F1 region in *Drosophila melanogaster* and several comments on the cytology of this region are appropriate. It is worth noting that from a γ-ray mutagenesis screen involving more than 8000 chromosomes, only one mutation, *Df(3L)Cat*^{DH104}, represented a confirmed cytological deficiency that extended into the 75D-E interval. Furthermore, similar mutagenesis experiments in other laboratories have failed to isolate cytological deficiencies for the 75D-E region (MICHAEL ABBOTT and JUDITH LENGYEL, personal communications). Segmental aneuploid studies have shown that this interval is not haplo-insufficient although monosomics for the 75D-76A region exhibit poor viability (LINDSLEY *et al.* 1972, LUBINSKY and BEWLEY 1979) and the low recovery frequency of deletions within the 75D-E interval could be due to the region *per se*. Alternatively, viability could be affected by a position effect caused by the deletion of genetic material of the 75 region that is adjacent to the minute *M(3)S34* in 76A (ASHBURNER *et al.* 1981). Mutagenesis experiments

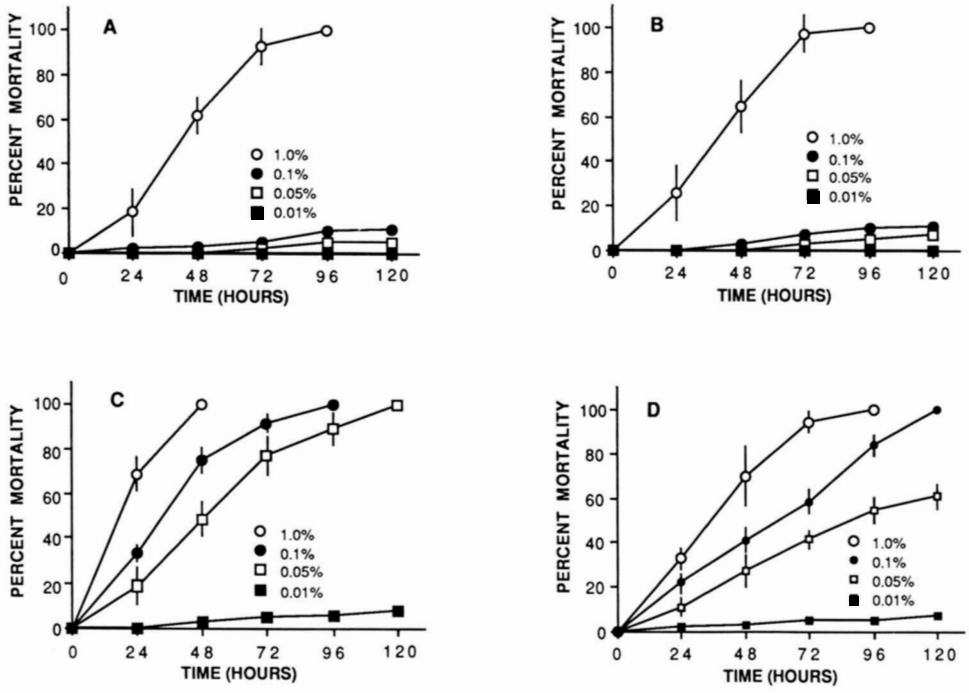

FIGURE 7.—The mortality rate of adult Drosophila when fed on differing concentrations of hydrogen peroxide. Triplicate cultures of fifty male or virgin female adults aged less than 24 hr were exposed to 1% (○), 0.1% (●), 0.05% (□), and 0.01% (■) hydrogen peroxide in shell vials containing 1% agar. Each culture was examined for survivors and transferred to fresh medium containing hydrogen peroxide daily for six days. Panel A represents the mortality rate of the wild type catalase stock. Panel B illustrates the mortality of the $Df(3L)Cat^{DH104}/Cat^{+}$ hemizygous strain. Panels C and D represent the mortality of the two mutant hemizygous strains, $Cat^{n1}/Df(3L)Cat^{DH104}$ and $Cat^{n2}/Df(3L)Cat^{DH104}$, respectively.

	+	n1	n2	n3	n4	n5	n6
+	100*	44	67	50	42	51	87
n1			4	2	0	1	11
n2				23	4	10	49
n3					2	1	51
n4						0	12
n5							20

FIGURE 8.—*Inter se* complementation matrix of the six catalase mutants for catalase activity. The activity of the multiply marked isogenic third chromosome strain *cp in ri* p^{p} was 152.4 ± 10.6 units/per mg protein (9 determinations). All other activities were presented as a percentage of the *cp in ri* p^{p} strain which was normalized to 100%. Shaded boxes represent heteroallelic combinations exhibiting significant positive complementation for catalase activity.

utilizing the alkylating agent diepoxybutane are currently in progress to obtain a series of smaller overlapping deficiencies within the interval uncovered by $Df(3L)Cat^{DH104}$ to further characterize this region and a complete cytogenetic analysis of the 75 region will be presented in a future report.

We have also isolated six acatalasemic mutants where two mutations, Cat^{n1} and Cat^{n4}, represent amorphic alleles of the catalase structural gene while the other four alleles are hypomorphic according to the definition of MULLER (1932). Complementation analysis of these mutant alleles has revealed several interesting observations. First, three alleles demonstrate positive intragenic complementation for catalase activity. It has been previously shown that the active enzyme is tetrameric in Drosophila (NAHMIAS and BEWLEY 1984), and therefore complementation can be attributed to a positive association between mutant/mutant and/or mutant/wild type polypeptide subunits creating a conformational change resulting in partial restoration of enzymatic activity (FINCHAM 1966). This now provides us with the genetic material to construct a series of strains with catalase activity levels ranging from 0 to 100% that can subsequently be used in studies designed to examine the protective role of catalase during conditions of oxygen stress. Second, the lack of any evidence for intergenic complementation between the six EMS-induced alleles strongly suggests that there is only one functional gene for catalase within the Drosophila genome. This is in contrast to a number of other systems including *E. coli* (LOEWAN *et al.* 1985), yeast (COHEN *et al.* 1985), Neurospora (SIMMONS, CHARY and NATVIG 1987) and maize (ROUPAKIAS, MCMILLIN and SCANDALIOS 1980) where genetic analysis has indicated the presence of multiple unlinked genes coding for catalase. Drosophila therefore represents an ideal eukaryotic system to study the phenotypic effects of acatalasemia since such studies would be uncomplicated by multiple genes exhibiting overlapping developmental and/or tissue specific expression.

Previous studies have mapped two naturally occurring variants, *MN18* and *RI33*, that alter both the temporal and tissue-specific expression of catalase to position 3–47.0 on *3L* (BEWLEY, MACKAY, and COOK 1986). Each variant was shown to differ in the rate constant for catalase synthesis (k_s) and this difference was correlated to different steady state levels of cata-

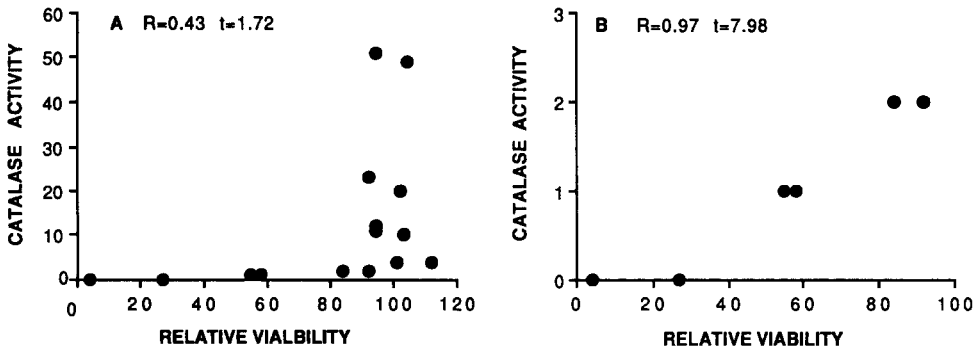

FIGURE 9.—Scatter plots illustrating the relationship between adult catalase activity and relative viability for the catalase heteroallelic crosses in Figure 8. Panel A shows the relationship between catalase activity and viability for all 15 heteroallelic combinations. Panel B is a subset of panel A illustrating the relationship between catalase activity and viability for heteroalleic combinations where the adult catalase activity is less than 3% of wild type. 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 viability, and t = the Student's t-test value. The relationship depicted in panel A is not significant at a p value of 0.10 while the relationship shown in panel B is significant at a p value less than 0.01.

lase translatable mRNA. In this study the EMS-induced mutation Cat^{n1} was also mapped to position 3–47.0. Based on these mapping studies, the temporal variants are tightly linked to the catalase structural gene as defined by the Cat^{n1} mutation. It is likely that these variants define a temporal element that regulates the developmental expression of catalase by altering the steady state level of Cat-specific mRNA.

Initial studies of all possible mutant heteroallelic combinations did not show a significant correlation between relative viability and adult catalase activity. However, upon closer examination very low levels of catalase activity (less than 2% of normal) have a severe negative viability effect suggesting that a threshold level of catalase activity exists above which viability is not affected. A reasonable explanation for such a threshold phenomenon is that catalase is known to be an extraordinarily efficient enzyme where the catalytic rate constant is approximately 10^7 liter mol^{-1} sec^{-1} (AEBI 1984), and it has been estimated that one molecule of catalase can decompose approximately 42,000 molecules of hydrogen peroxide per second at 0° in mammalian tissues (AEBI and WYSS 1978). Thus it appears that low levels of catalase are sufficient to minimize the cytotoxic effects of H_2O_2. This finding is particularly interesting when viewed in the light of earlier reports which describe genetic defects that influence catalase expression in mammalian systems. Human acatalasemics have been reported to exhibit a deficiency in blood catalase levels while catalase in solid tissues is only slightly affected (TAKAHARA 1952, 1968). Short term clinical manifestations of human acatalasemia occur predominantly in the mouth with the development of oral ulcerations; however, no long term health effects have been reported. The only other reported mammalian acatalasemic mutation was isolated in mice by FEINSTEIN *et al.* (1966). We have reexamined this strain and have shown that while erythrocyte catalase is severely reduced, the acatalasemic phenotype is expressed to a different extent in kidney and liver tissue, *i.e.*, 16% and 90%, respectively, of the same tissues in normal mice (SHAFFER, SUTTON, and BEWLEY 1987; BEWLEY and MACKAY 1989). No phenotypic effects have been associated with this mouse strain. The threshold effect described for the Drosophila acatalasemics provides a reasonable explanation for the lack of observable phenotypic effects in the mammalian acatalasemics since catalase for both the human and mouse mutations could be expressed at sufficient levels in solid tissues to afford protection from the long term cytotoxic effects of endogenous H_2O_2.

Although essential for cellular metabolism in aerobic organisms, dioxygen has long been known to be a toxic agent which under appropriate conditions can result in considerable cellular damage. It was subsequently postulated that oxygen free radical derivatives of the reduction pathway for dioxygen are the responsible agents in the toxic effects of oxygen, and not ground state molecular oxygen (MCCORD, KEELE and FRIDOVICH 1971; FRIDOVICH 1977). Although H_2O_2 is technically not a free radical, it is a strong oxidant capable of rapidly diffusing through cytological membranes from its endogenous site of origin and it is well established as a potent agent involved in both cell death and mutagenic events in *E. coli* (HASSAN and MOODY 1984; FARR, D'ARI and TOUATI 1986; IMLAY and LINN 1988). In the presence of reduced transition metals H_2O_2 is further reduced in a superoxide-driven Fenton cycle to the hydroxyl radical which reacts at nearly diffusion-limited rates with most organic substrates (GREEN and HILL 1984). Catalase and other

peroxidases have long been postulated to represent a main line of defense against oxygen free radical induced damage by scavenging H_2O_2 and thereby limiting formation of the highly reactive hydroxyl radical. However, it has not been practical to directly test this idea in multicellular eukaryotes due to the general unavailability of appropriate genetic material. The fact that loss of function mutants for catalase in Drosophila are hypersensitive to H_2O_2 and exhibit severe viability effects provides strong evidence that catalase does represent a major pathway for scavenging endogenous H_2O_2 generated in eukaryotic cells. These results are consistent with those obtained for both *E. coli* and yeast mutants in suggesting that catalases in general provide significant protection against the toxic effects of oxygen free radical induced cellular damage. We are particularly interested in using this collection of mutants to examine the mechanism and rate of oxygen radical induced genetic damage, the relationship of increasing levels of genetic damage to aging and life-span parameters, and the protective role of catalase and other antioxidant enzymes in these processes.

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