TEMPORAL VARIATION FOR THE EXPRESSION OF CATALASE IN *DROSOPHILA MELANOGASTER*: CORRELATIONS BETWEEN RATES OF ENZYME SYNTHESIS AND LEVELS OF TRANSLATABLE CATALASE-MESSENGER RNA

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ABSTRACT

Two variants that alter the temporal expression of catalase have been isolated from a set of third chromosome substitution lines. Each variant has been mapped to a cytogenetic interval flanked by the visible markers *st* (*3*-44.0) and *cu* (*3*-50.0) at a map position of 47.0, which is within or near the interval 75D-76A previously identified as containing the catalase structural gene on the bases of dosage responses to segmental aneuploidy. Each variant operates by modulating the rate of enzyme synthesis and the level of translatable catalase-mRNA.

AEROBIC organisms generate highly reactive free radicals as a by-product of oxygen reduction via both enzymatic and nonenzymatic reactions (FRIDOVICH 1977; AMES 1983; HALLIWELL and GUTTERIDGE 1984; CERUTTI 1985). Oxygen free radicals are highly reactive in biological systems and are capable of disrupting the structural and functional integrity of the cell through lipid peroxidation events, DNA damage and protein inactivation (AMES 1983; CERUTTI 1985; GREENSTOCK 1984; MEAD 1984; BURTON and INGOLD 1984). The biological consequences of free radical damage are numerous, including mutations, sister chromatid exchanges, chromosomal aberrations, cytotoxicity, carcinogenesis, and cellular degeneration related to aging (CERUTTI 1985; KENSLER and TRUSH 1984; HARMAN 1984). Substantial evidence exists in the literature for a form of intracellular enzymatic defense against free radical intermediates (FRIDOVICH 1977; AMES 1983; HALLIWELL and GUTTERIDGE 1984; CERUTTI 1985). To gain an understanding of the relationship between free radical damage and the manifestation of disease, the defense mechanisms active in this process must be examined in detail. Efforts in this laboratory are concentrating on one aspect of this problem, the molecular organization and genetic regulation of enzymes involved in oxygen radical metabolism.

We have chosen the gene-enzyme system catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) as a first step in this analysis for two reasons. First, catalase is a major antioxidant enzyme involved in the protective destruction of hydro-

gen peroxide generated during cellular metabolism, ionizing radiation and the dismutation of superoxide radicals (MASTERS and HOLMES 1977; CHANCE, HELMUT and BOVERIS 1979; BEWLEY and LUBINSKY 1980; STARKE and FARBER 1985). Second, simple but sensitive techniques have been developed for measuring the kinetics of catalase turnover *in vivo* (PRICE, RECHCIGL and HARTLEY 1961; PRICE *et al.* 1962). The compound 3-amino-1,2,4-triazole (AT) is a noncompetitive inhibitor that binds to the protein apoenzyme of catalase to form an irreversible complex (MARGOLIASH, NOVGRODSKY and SCHEJTER 1960; REID *et al.* 1981). This complex results in the destruction of existing catalase molecules without interfering with *de novo* synthesis. Thus, the initial rate of reappearance of catalase activity directly reflects the rate of *de novo* synthesis and allows estimation of the rate constants for both synthesis and degradation (RECHCIGL 1968; FRITZ and PRUITT 1977).

The structural gene, Cat^+, for Drosophila catalase has been mapped to the cytogenetic region 75D–76A on the left arm of chromosome 3 on the basis of dosage responses to segmental aneuploidy (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984a). The enzyme has been purified to homogeneity and is tetrameric with a subunit molecular mass of 58 kD, and catalase-monospecific antibodies have been raised (NAHMIAS and BEWLEY 1984b). In a previous study utilizing a large sample of chromosomes isolated from natural populations, significant line-by-stage interactions were observed for the third chromosome substitution lines (BEWLEY and LAURIE-AHLBERG 1984). In the present study, several of these lines have been selected for a more detailed examination of the genetic variation affecting the temporal expression of the catalase gene. The ultimate goals of this project are to identify genetic elements that program the temporal and tissue-specific expression of catalase, to gain insight into the range of effects these elements have on modulating rates of enzyme synthesis and degradation and to develop material suitable for investigating the molecular mechanisms whereby these processes exert their effects.

EXPERIMENTAL PROCEDURES

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

The lines described in this study were derived from natural populations. The constitution of a line is i_1/i_1; i_2/i_2; $+_3/+_3$ (referred to as a third chromosome substitution line), where *i* refers to a chromosome from a highly inbred line (Ho-R) and + refers to a chromosome isolated from a natural population. The + but not the *i* chromosomes vary between lines, and therefore each established line differs in only the third chromosome, while remaining coisogenic for chromosomes *1* and *2*. The details of line construction and karyotypic analysis have been described (LAURIE-AHLBERG *et al.* 1980; LAURIE-AHLBERG *et al.* 1982).

The mapping of activity level variation among the third chromosome substitution lines made use of a multiply marked third chromosome line, *ru cu ca*, that was in the same isogenic background. The third chromosome markers

were *ru* (3-0.0), *h* (3-26.5), *th* (3-43.2), *st* (3-44.0), *cu* (3-50.0), *sr* (3-62.0), *e* (3-70.7) and *ca* (3-100.7). A *ru Pri ca* chromosome contained the same recessive mutations as *ru cu ca* in addition to the dominant marker *Pri* (3-90.0). These stocks were provided by C. C. LAURIE-AHLBERG. All genetic symbols are described in LINDSLEY and GRELL (1968).

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

Catalase activity was determined by one of two methods. A polarographic assay using a Clark-type oxygen probe was used to determine activity as a function of development (GOLDSTEIN 1968). One unit of activity is defined as the release of 1 μmol O_2/min. All other assays were conducted by the spectrophotometric method of BEERS and SIZER (1952), and this method has been described in detail for Drosophila (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984b). The disappearance of H_2O_2 was monitored at 230 nm, where one unit of activity is defined as 1 μmol of H_2O_2 decomposed per minute, based on a molar absorptivity for H_2O_2 of 62.4 (NELSON and KIESOW 1972). Protein determinations were conducted by the dye-binding method of BRADFORD (1976).

Developmental staging and tissue dissection: Synchronous cultures were established as previously described (BEWLEY, NAHMIAS and COOK 1983), using morphological criteria for pupae as described by BAINBRIDGE and BOWNES (1981).

All larvae were collected within 2 hr of hatching and were dissected as third instars 120 ± 3 hr later. Newly emerged imagos were collected within a 6-hr period after eclosion and were isolated by sex for 48 hr. The details of organ and tissue dissection for each developmental stage have been described (BEWLEY, NAHMIAS and COOK 1983).

Immunological procedures: The purification of Drosophila catalase to homogeneity by hydrophobic interaction chromatography and the subsequent production of monospecific catalase antibodies have been described (NAHMIAS and BEWLEY 1984b).

Ouchterlony double diffusion and quantitative rocket immunoelectrophoresis of catalase antigen have been described in detail (BEWLEY, NAHMIAS and COOK 1983). Since the catalase antigen-antibody complex retains enzymatic activity, the gels were stained for activity using a ferric chloride-potassium ferricyanide solution (WOODBURY, SPENCER and STAHMAN 1971). The area under each rocket was approximated by multiplying the rocket height in millimeters by the rocket width at half-height. A standard calibration curve was linear with serial dilutions of crude antigen.

The immunoprecipitation of ^{35}S-labeled catalase from *in vivo* radiolabeled flies or from the *in vitro* translation of poly(A)$^+$ RNA followed a protocol previously described (SHAFFER and BEWLEY 1983).

Western blots: Western-blot analysis was conducted as described (TOWBIN, STAEHELIN and GORDON 1979). Total proteins from crude extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted

onto nitrocellulose. The filter was overlaid first with partially purified anti-catalase IgG and second with goat-anti rabbit IgG conjugated with horseradish peroxidase according to instructions provided by the supplier (BioRad).

Substrate kinetics and pH optima determinations of the catalase protein were conducted as previously described (NAHMIAS and BEWLEY 1984b).

Dietary administration of 3-amino-1,2,4-triazole: Adult male flies aged for 5 days posteclosion were nutritionally deprived for 12 hr on 2.5% agar. Flies were subsequently transferred without etherization to agar bottles containing Kimwipes saturated with 3 ml of a 5 mM aminotriazole solution in 2% sucrose. Control flies were fed with the sucrose solution in the absence of aminotriazole. Flies were allowed to feed for 6 hr and were then placed onto chase media until sacrificed.

Analysis of turnover parameters: Turnover parameters were determined by the aminotriazole perturbation method of PRICE, RECHCIGL and HARTLEY (1961) and PRICE et al. (1962) as described for Drosophila (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984b). At specific time intervals following an aminotriazole feeding, flies were sacrificed and the level of catalase activity was determined. The log of the difference between enzyme activity at the steady state (C_N) and activity at time intervals during the recovery phase (C_t) was plotted as a function of time. The rate constants for degradation (k_d) were estimated as the slope of the linear regression of $\ln(C_N - C_t)$ on t according to the relationship

$$K_d = \left[\ln \frac{C_N}{C_N - C_t}\right] \div t.$$

The rate constants for synthesis (k_s) were estimated from the relationship $k_s = k_d \cdot C_N$, and half-life values for catalase were estimated from $t_{1/2} = \ln 2/k_d$ (SCHIMKE 1975).

***In vivo* radiolabeling:** The *in vivo* radiolabeling of Drosophila catalase by microinjection has been thoroughly described (WILKENS, SHAFFER and BEWLEY 1982). Each fly received approximately 0.357 μCi [^{35}S]methionine per injection (New England Nuclear; specific activity, 1000 Ci/mmol).

RNA isolation: Total RNA was purified from adult Drosophila by guanidinium thiocyanate extraction (CHIRGWIN *et al.* 1979). Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT) cellulose (Collaborative Research, Inc.). The integrity of each RNA preparation was analyzed by electrophoresis on methylmercury hydroxide agarose gels and by an analysis of total translation products by SDS-PAGE and fluorography.

***In vitro* translation:** *In vitro* translation of Drosophila poly(A)$^+$ RNA was performed using a rabbit reticulocyte translation kit (New England Nuclear). Reaction mixtures of 50 μl contained 20 μl of rabbit reticulocyte lysate with all recommended translation kit components supplemented with 60 μmol of potassium acetate and 400 nmol of magnesium acetate. Poly(A)$^+$ RNA was added to a final concentration of 2 μg per assay, and 100 μCi of [^{35}S]methionine was used in each assay. After incubation at 37° for 60 min, 5-μl aliquots were removed for trichloroacetic acid precipitation and for analysis of total

translation products by SDS-PAGE and fluorography. The translational assay was linear for total TCA precipitable counts for up to 5 μg of poly(A)$^+$ RNA.

Electrophoresis and fluorography: Total translation products and immunoprecipitated catalase were electrophoresed in 12.5% SDS polyacrylamide slab gels as described (WRIGHT, SHAFFER and BEWLEY 1985). For fluorography, gels were treated with Enhance (New England Nuclear) following the manufacturer's directions, dried onto Whatman No. 1 filter paper and exposed to Kodak X-Omat film at −70° for 24–48 hr.

RESULTS

Quantitative activity level variation: We have investigated several aspects of catalase activity variation using a well-defined set of lines in which a large sample of third chromosomes isolated from natural populations were substituted into a co-isogenic background (LAURIE-AHLBERG *et al.* 1980; BEWLEY and LAURIE-AHLBERG 1984). This set of lines has proven to be a rich source of variation for the quantitative expression of catalase. In addition, experiments were performed that suggested that stage-specific genetic effects exist between lines. We have subsequently selected two lines that exhibited rather large differences in line by stage interaction for a more detailed analysis.

Developmental program: The developmental expression of catalase has been previously described (BEWLEY, NAHMIAS and COOK 1983). Two distinct peaks of activity are observed during Drosophila development 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 newly eclosed adults that is approximately one-third the previous maximum value and that is maintained throughout the remainder of the adult life cycle. A convenient way of representing the temporal expression of Drosophila catalase is by a ratio of activity levels for each of the two major peaks (*i.e.*, peak-I/peak-II). For most lines that are examined, this activity ratio varies from 0.70–0.85 and has been designated as the wild-type temporal pattern.

Two variant lines of the wild-type temporal pattern are illustrated in Figure 1. While each variant is both quantitatively and qualitatively different, they are similar to wild-type patterns in the timing of expression of each activity peak. Line RI33 exhibits a general quantitative reduction in catalase expression throughout development with an activity ratio of 0.47, indicating a disproportionate reduction of peak I. In contrast, line MN18 is a high activity line, where peak I is overexpressed yielding an activity ratio of 1.5 (Figure 1a). Catalase activity subsequently reaches a steady state level in newly eclosed adults with a twofold quantitative difference being maintained between each line.

Tissue-specific expression: The expression of catalase in the tissues of both adult and larval stages of development in lines RI33 and MN18 is illustrated in Figure 2. Catalase activity is found at varying levels in all tissues examined and is therefore ubiquitous in its expression. Line MN18 exhibits a greater

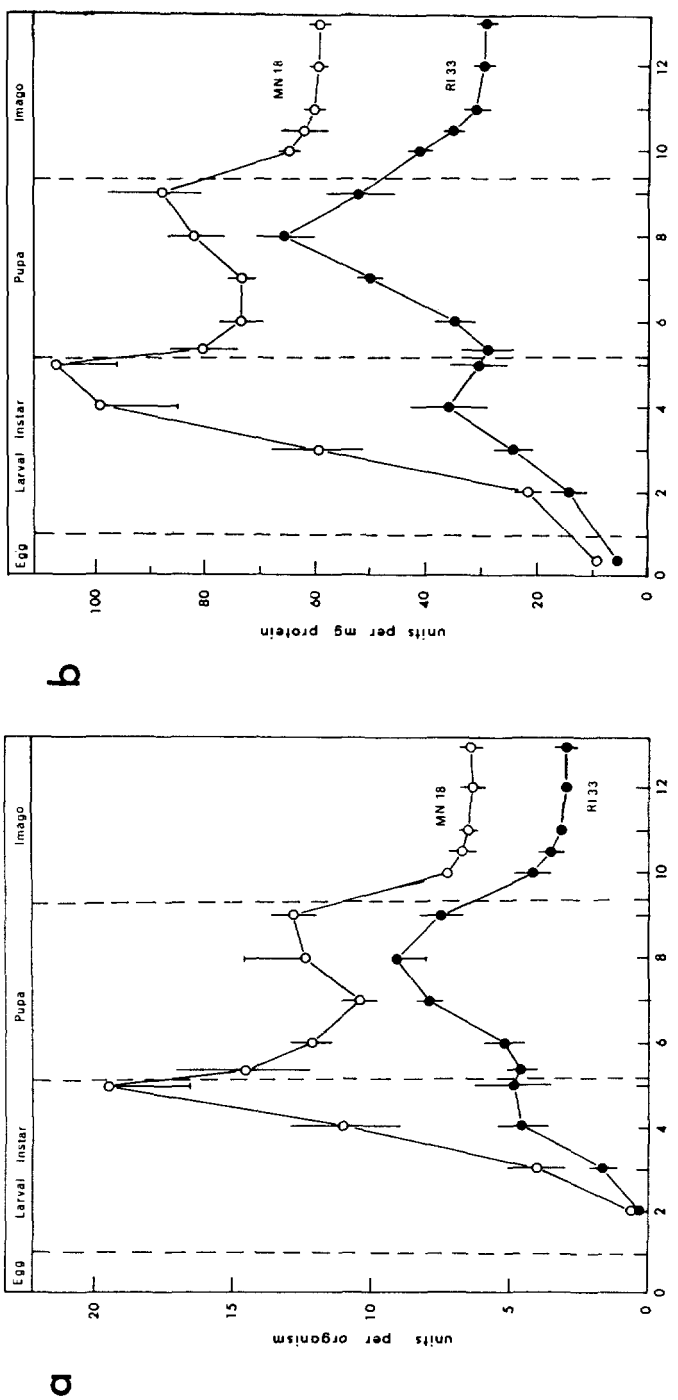

FIGURE 1.—Developmental expression of catalase activity in two different third-chromosome substitution lines. Each point is the mean of three determinations ± one standard deviation where (○—○) = MN18 and (●—●) = RI33. a, Activity expressed as units per individual at each developmental stage; b, activity expressed as units/milligram protein.

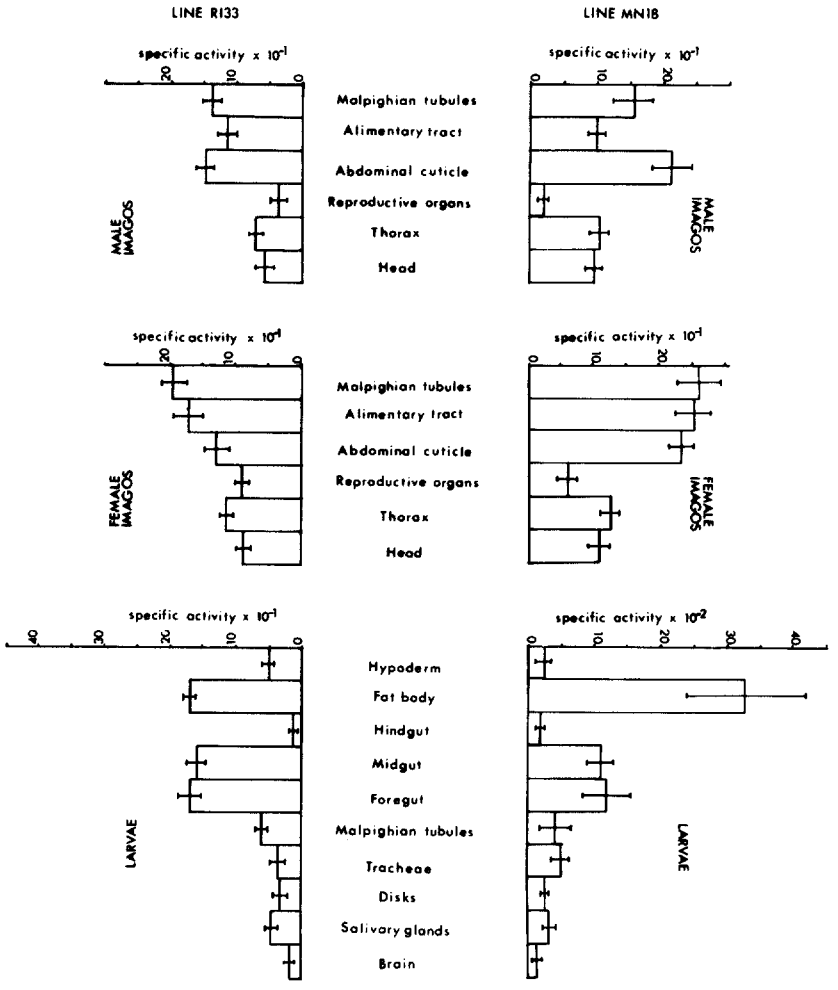

FIGURE 2.—The tissue-specific expression of catalase in the lines RI33 and MN18. All samples represent the mean specific activity ± the 95% confidence interval determined from four separate extracts.

activity level in most tissues when compared with line RI33, which is consistent with assays conducted on whole flies and larvae. Of particular interest is a 19- to 20-fold difference in the level of catalase expression in larval fat body of line MN18 that is the major tissue-specific effect between lines and that can account for the unusually large peak of catalase overproduction in late third-instar larvae of line MN18 (Figure 1).

Genetic control of activity: Cytological analysis indicated that the third chromosome from both lines RI33 and MN18 are normal. Segregation analysis of F_1 and backcross progeny for crosses between RI33 and MN18 indicated that the catalase activity level variation segregates as a single genetic site with additive inheritance (data not shown).

The genetic crosses outlined in Figure 3 were subsequently utilized to map

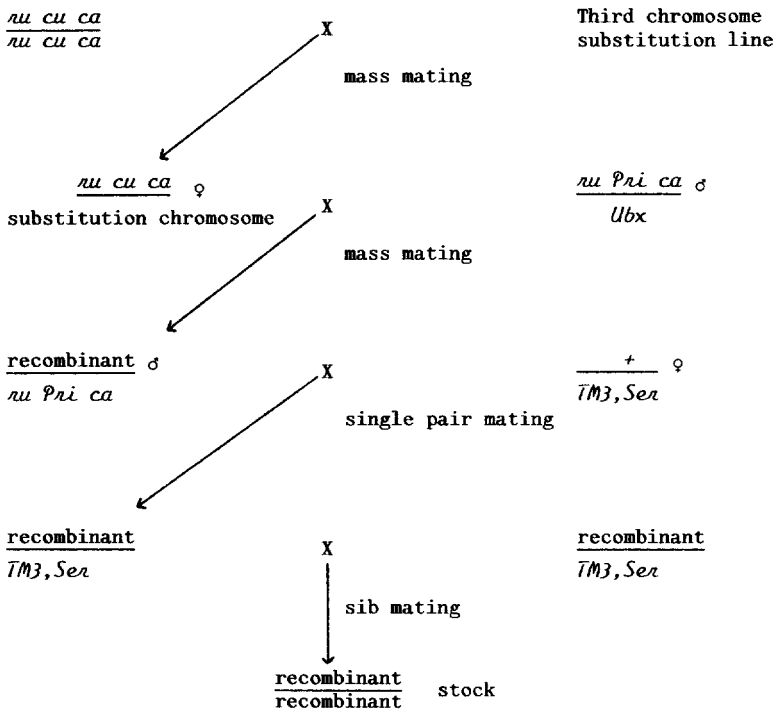

FIGURE 3.—Crossing scheme used to localize the site responsible for quantitative variation in catalase activity in lines RI33 and MN18. All genetic symbols and special chromosomes are described in EXPERIMENTAL PROCEDURES or in LINDSLEY and GRELL (1968).

the site responsible for catalase activity variation relative to visible markers on the multiply marked third chromosome *ru cu ca*. Out of a total of 2089 male progeny, 1390 RI33 recombinant males were isolated and were single-pair mated. The recombinant chromosomes represented all possible single recombination events defined by the *ru cu ca* markers. Ten separate events for each interval were subsequently assayed as homozygotes to initially localize the low activity site in line RI33. Table 1 clearly demonstrates that this site lies between the visible markers *st* (*3*-44.0) and *cu* (*3*-50.0). Subsequently, homozygotes representing 22 separate recombination events between *st* and *cu* were assayed (Table 1) to give a calculated map position for RI33 of *3*-47.0 with a 95% confidence interval of ±0.5.

A similar experiment was conducted on line MN18 with the following modification. Since the *ru cu ca* chromosome exhibits relatively high levels of catalase activity compared to MN18 (Tables 1 and 2), the site responsible for underproduction in line RI33 was crossed into the *ru cu ca* chromosome to facilitate phenotypic identification of recombinant progeny based on catalase activity levels (Figure 3). Thus line MN18 was mapped relative to the *ru cu RI33 ca* underproducer chromosome. This experiment clearly placed the site controlling overproduction of catalase between the interval *st* and *cu* with a map position of *3*-46.7 ± 0.8 (Table 2). Therefore each site responsible for

TABLE 1

Genetic mapping of catalase underproduction in line RI33

(A)

Parental genotype	Catalase activity[a]	Phenotype
ru h th st cu sr e ca / *ru h th st cu sr e ca*	100[b]	Normal
(*ru cu ca*) *RI33* / *RI33*	56[c]	Underproducer

(B)

Recombinant genotype	Catalase activity	Phenotype	Recombination event	No. of events assayed
ru / *ru*	60	Underproducer	*ru* and *h*	5
h th st cu sr e ca / *h th st cu sr e ca*	106	Normal	*ru* and *h*	5
ru h / *ru h*	57	Underproducer	*h* and *th*	5
th st cu sr e ca / *th st cu sr e ca*	89	Normal	*h* and *th*	5
ru h th / *ru h th*	59	Underproducer	*th* and *st*	5
st cu sr e ca / *st cu sr e ca*	99	Normal	*th* and *st*	5
ru h th st / *ru h th st*	57	Underproducer	*st* and *cu*	9
ru h th st / *ru h th st*	110	Normal	*st* and *cu*	6
cu sr e ca / *cu sr e ca*	101	Normal	*st* and *cu*	5
cu sr e ca / *cu sr e ca*	53	Underproducer	*st* and *cu*	2
ru h th st cu / *ru h th st cu*	109	Normal	*cu* and *sr*	5
sr e ca / *sr e ca*	57	Underproducer	*cu* and *sr*	5
ru h th st cu sr / *ru h th st cu sr*	97	Normal	*sr* and *e*	5
e ca / *e ca*	66	Underproducer	*sr* and *e*	5
ru h th st cu sr e / *ru h th st cu sr e*	99	Normal	*e* and *ca*	5
ca / *ca*	51	Underproducer	*e* and *ca*	5

[a] All enzyme activities are presented as a percentage of activities, with *ru cu ca* as 100%.

[b] The enzyme activity of the multiply marked isogenic third chromosome strain *ru cu ca* was 148.2 ± 13.3 units/mg protein (27 determinations).

[c] The enzyme activity of the third chromosome underproducer strain RI33 was 83.0 ± 5.8 units/mg protein (15 determinations).

TABLE 2

Genetic mapping of catalase overproduction in line MN18

(A)

Parental genotype	Catalase activity[a]	Phenotype
MN18 / *MN18*	100[b]	Overproducer
ru h th st RI33 cu sr e ca / *ru h th st RI33 cu sr e ca*	46[c]	Underproducer

(B)

Recombinant genotype	Catalase activity	Phenotype	Recombination event	No. of events assayed
ru / *ru*	94	Overproducer	*ru* and *h*	5
h th st cu sr e ca / *h th st cu sr e ca*	46	Underproducer	*ru* and *h*	5
ru h / *ru h*	100	Overproducer	*h* and *th*	5
th st cu sr e ca / *th st cu sr e ca*	41	Underproducer	*h* and *th*	5
ru h th / *ru h th*	98	Overproducer	*th* and *st*	5
st cu sr e ca / *st cu sr e ca*	42	Underproducer	*th* and st	5
ru h th st / *ru h th st*	48	Underproducer	*st* and *cu*	4
ru h th st / *ru h th st*	98	Overproducer	*st* and *cu*	3
cu sr e ca / *cu sr e ca*	47	Underproducer	*st* and *cu*	4
cu sr e ca / *cu sr e ca*	95	Overproducer	*st* and *cu*	5
ru h th st cu / *ru h th st cu*	47	Underproducer	*cu* and *sr*	5
sr e ca / *sr e ca*	114	Overproducer	*cu* and *sr*	5
ru h th st cu sr / *ru h th st cu sr*	51	Underproducer	*st* and *e*	5
e ca / *e ca*	93	Overproducer	*sr* and *e*	5
ru h th st cu sr e / *ru h th st cu sr e*	50	Underproducer	*e* and *ca*	5
ca / *ca*	102	Overproducer	*e* and *ca*	5

[a] All enzyme activities are presented as a percentage of activities, with *MN18* as 100%.

[b] The enzyme activity of the third chromosome overproducer strain MN18 was 179.3 ± 7.3 units/mg protein (24 determinations).

[c] The enzyme activity of the multiply marked isogenic third chromosome underproducer strain *ru RI33 cu cu* was 83.0 ± 5.8 units/mg protein (15 determinations).

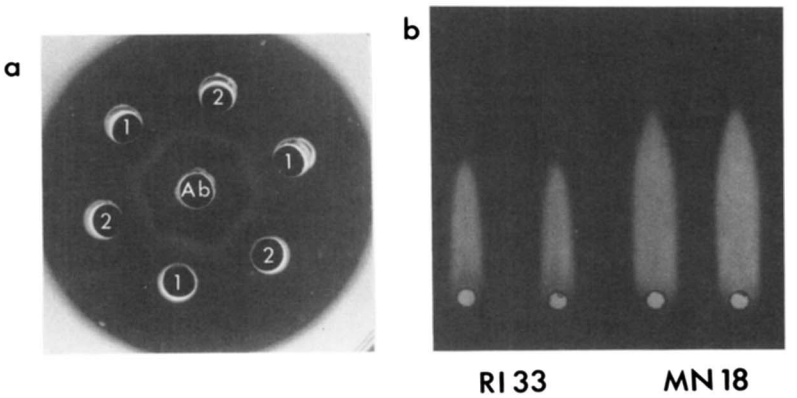

FIGURE 4.—Immunological analysis of Drosophila catalase. a, Ouchterlony double-diffusion plate demonstrating antigenic similarity of catalase antigen from lines RI33 (1) and MN18 (2). The center well contained 20 μl of antiserum. The gel was stained for catalase activity using a ferric chloride-potassium ferricyanide solution (WOODBURY, SPENCER and STAHMAN 1971). b, The steady state level of CAT-specific CRM in 5-day-old adult flies as determined by rocket immunoelectrophoresis. Each sample and the mean relative area under the rockets are as follows: RI33 (38.7 mm^2) and MN18 (96.2 mm^2).

catalase activity variation maps within an interval on the left arm of chromosome *3* that is in or near the interval 75D–76A, which has previously been shown to elicit a dosage response for catalase expression with segmental aneuploidy (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984a).

Characterization of steady-state protein: The antigenic specificity of catalase protein from lines RI33 and MN18 was evaluated by Oucherlony double immunodiffusion experiments (Figure 4a). A single confluent precipitin line was evident on double diffusion plates for crude extracts of each line, suggesting identical antigenic determinants for each protein. The steady state level of catalase-specific protein in male flies 5 days postemergence was examined by rocket immunoelectrophoresis, where line MN18 exhibits 2.5 times the catalase-specific cross-reacting material (CRM) relative to RI33 (Figure 4b). In addition, we have demonstrated that CRM levels as a function of development parallel the enzyme activity level data in Figure 1 (data not shown).

In order to more completely assess the structural relatedness of catalase protein from each line, several physicochemical and kinetic parameters were examined. Crude extracts from each line exhibited the same broad pH optimum for catalase activity, pH 6.5–7.5, and the same affinity for the substrate H_2O_2, Kapp = 45.9–46.7 mM. Western blot analysis illustrated that there is one form of the catalase protein in both the larval and adult stages of development, each with an M_r = 58,000 (Figure 5). These results, in addition to the immunological data, suggests that the active site is conserved for each protein and that the quantitative differences in activity level are not due to altered kinetic properties but, rather, to differential rates of accumulation of catalase protein.

Analysis of catalase turnover: Variation in the rates of accumulation of catalase-specific protein (CRM) must be explained by differential rates of en-

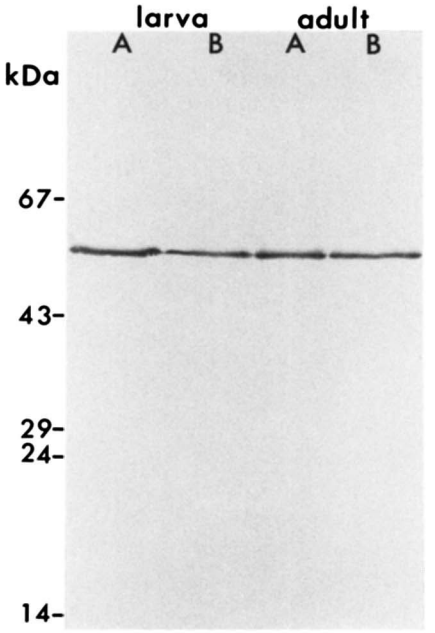

FIGURE 5.—Western blot of total Drosophila proteins separated on 12.5% SDS PAGE and electroblotted onto nitrocellulose. Larval and adult samples were homogenized at a concentration of one individual per 50 μl and 10 μl of each extract was applied to the gel. Lanes A, line MN18; lanes B, line RI33. The nitrocellulose filter was overlaid first with partially purified catalase antibodies and second with goat-anti rabbit IgG conjugated with horseradish peroxidase.

zyme synthesis, intracellular degradation or a combination of these two processes. Aminotriazole perturbation of catalase activity provides an effective method for determining turnover parameters in the adult fly, since steady state levels are attained within 12–24 hr of eclosion and are maintained essentially unchanged throughout the adult life cycle. However, this inhibitor is not useful for earlier stages of development, because it is toxic (LUBINSKY and BEWLEY 1979) and because catalase activity does not reach a steady state level (Figure 1). Since it was impractical to use this inhibitor for larval tissues, this analysis was restricted to adults only.

The kinetics of catalase destruction *in vivo* by the dietary administration of 5 mM aminotriazole is illustrated in Figure 6. It is apparent from this experiment that the uptake of the inhibitor and subsequent inactivation of existing catalase molecules is very effective with rates that are similar between the two lines. Approximately 50% of the catalase activity is destroyed within 1 hr of feeding, and complete inactivation is obtained by the end of a 6-hr feeding.

The rate of return of catalase activity to steady state levels in lines RI33 and MN18 is illustrated in Figure 7a. The clearance of residual aminotriazole from the tissues of adult Drosophila occurs within 12 hr of termination of feeding on the inhibitor, as evidenced by the lack of a significant time lag within the first 24 hr of *de novo* catalase synthesis (Figure 7a) and by the linearity of the plot of $\ln(C_N - C_t)$ on t (Figure 7b). In addition, the pattern

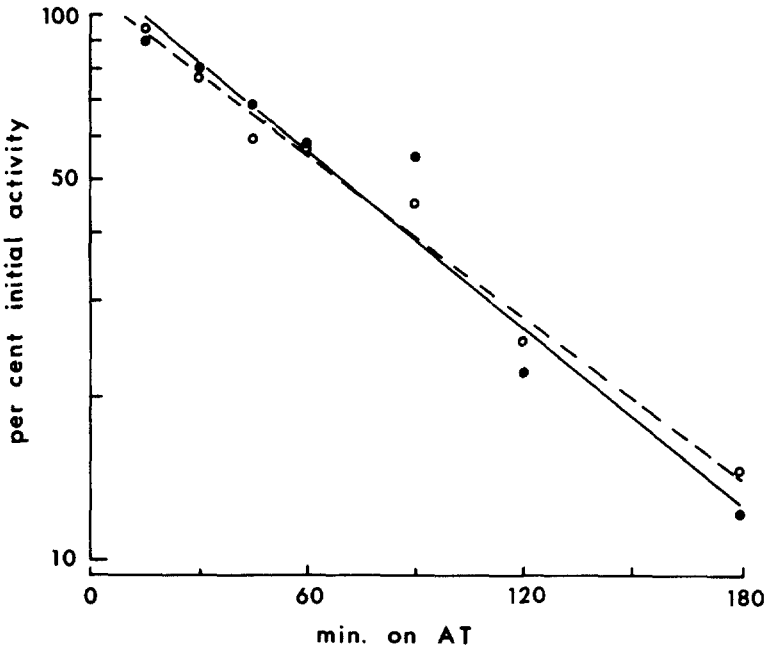

FIGURE 6.—The kinetics of catalase destruction *in vivo*. The destruction of catalase in adult male flies of the third chromosome—substitution lines MN18 (O——O) and RI33 (●—●)—upon the dietary administration of 5 mM 3-amino-1,2,4-triazole (AT). Each point is the mean of three separate determinations. Each line was fitted by regression analysis ($r > 0.98$) with slope −0.3273 (●—●) and −0.2985 (O——O).

of *de novo* catalase synthesis follows the same time course to steady state levels for each line, with MN18 reaching a level that is twofold greater than that for RI33. These results suggest that the relative rate of catalase synthesis is greater in line MN18.

The first order kinetics of catalase degradation *in vivo* is illustrated in Figure 7b. The slope of each plot of $\ln(C_N - C_t)$ on t is equal to $-k_d$ (FRITZ and PRUITT 1977). The plots obtained for each strain of flies are parallel, indicating that there is no significant difference in the rate of catalase degradation. Estimated turnover parameters are summarized in Table 3. These results suggest that the differences in the rate of catalase accumulation in the two fly lines result from differential rates of enzyme synthesis.

Cell-free translation of catalase mRNA: *In vitro* translation of poly(A)$^+$ RNA isolated from both lines RI33 and MN18 followed by immunoprecipitation of *de novo* synthesized catalase is illustrated in Figure 8. The *in vitro* translation product has a molecular mass of 58 kD, which is identical to the product labeled *in vivo* by microinjection of adult flies and to purified Drosophila catalase used as a marker protein. These results corroborate the protein blotting data of Figure 5 in suggesting that catalase is expressed as a single molecular form during Drosophila development.

Figure 8 also demonstrates that the level of translatable CAT-mRNA in line MN18 is greater than that found in line RI33. This has been confirmed by

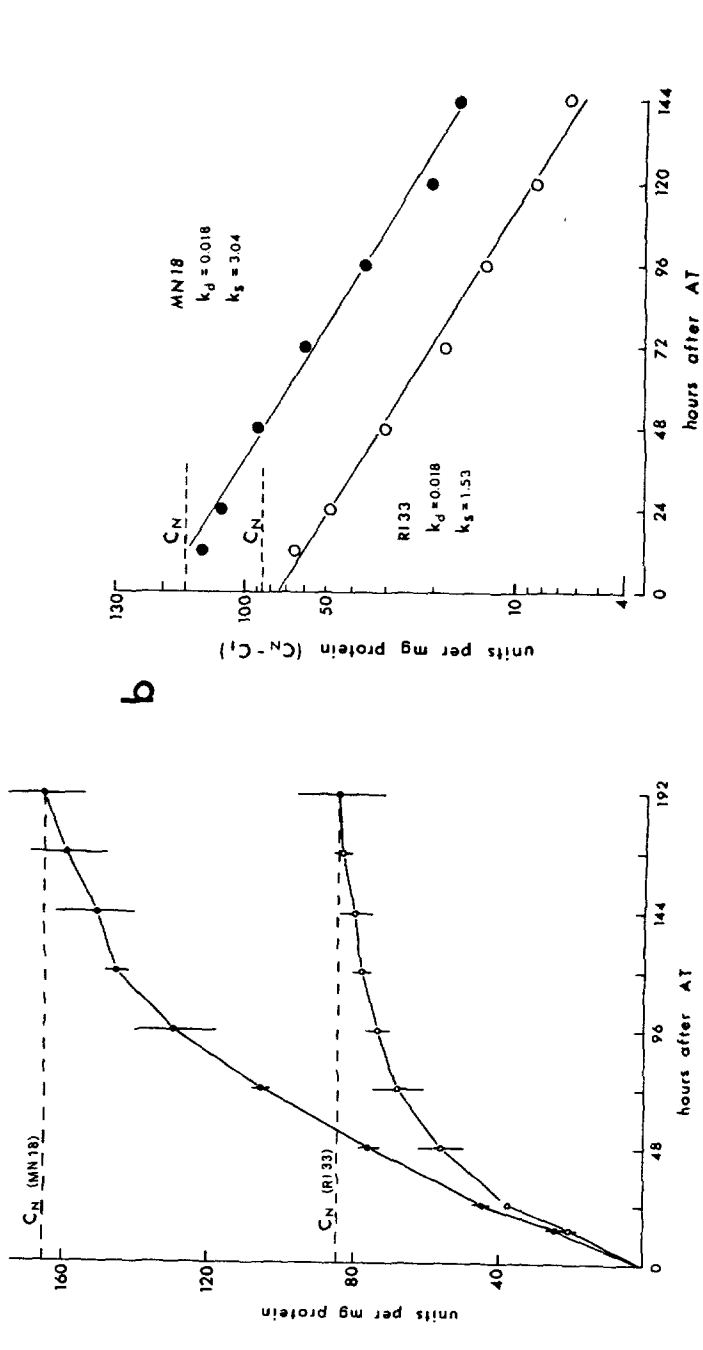

FIGURE 7.—*The kinetics of catalase turnover in vivo. a*, The return of catalase activity to steady state levels following the dietary administration of 3-amino-1,2,4-triazole to lines MN18 and RI33. C_N is the steady state level determined from flies fed on a sucrose control solution. Each point is the mean of three separate determinations ± one standard deviation. b, A semilogarithmic plot of data in Figure 8a presented as $C_N - C_t$ *vs.* time, where C_N is the steady state level and C_t is the catalase level at time intervals after the administration of AT. Each line was fitted by regression analysis with $r > 0.99$.

TABLE 3

Summary of turnover parameters for catalase

Line	k_d[a]	k_s[b]	C_N[c]	Half-life
RI33	0.018	1.53	86.1 ± 0.81	38.7
MN18	0.018	3.04	168.8 ± 0.41	38.1

[a] k_d represents the constant fraction of catalase molecules destroyed per hour.

[b] k_s represents the units of catalase activity synthesized per hour (units/milligram protein per hour).

[c] C_N represents the mean steady state level of catalase activity in adult flies determined at the beginning and end of each experiment and given as units/milligram protein ± SD.

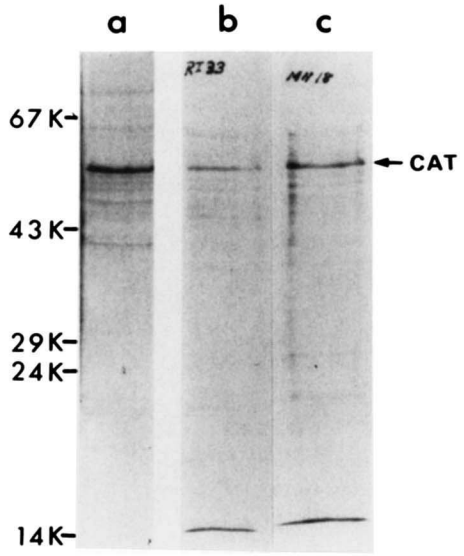

FIGURE 8.—SDS-PAGE and fluorography of ^{35}S-labeled catalase recovered by immunoprecipitation. a, Immunoprecipitable catalase labeled *in vivo* by microinjection of 0.357 μCi of [^{35}S]-methionine into each of 15 adult flies of line MN18 following a 6-hr incorporation period. b and c, Immunoprecipitable catalase recovered from an *in vitro* translation of poly(A$^+$) RNA isolated from line RI33(b) and line MN18(c). Each translation assay was made equivalent with respect to total trichloroacetic acid precipitable counts, *i.e.*, approximately 262,912 cpm, before immunoprecipitation.

quantitative densitometric tracings of each band (RI33/MN18 band ratio = 0.643). Therefore, the twofold differences in the rate of enzyme synthesis between these two lines appears to be related to different steady state levels of translatable CAT-mRNA.

DISCUSSION

The results reported here confirm our earlier observations of significant stage-specific effects for catalase expression within a set of third chromosome substitution lines (BEWLEY and LAURIE-AHLBERG 1984). The most striking tem-

poral effect is the relationship between the two major peaks of expression during development. Peak I is overexpressed in line MN18 and underexpressed in RI33 when compared to peak II of each line. Since CRM levels in each line parallel the temporal patterns for enzyme activity, each peak of expression must result in an alteration of the rate of accumulation of catalase protein. A question of primary importance is whether each variant alters the steady state level of catalase protein by changing rates of enzyme synthesis, intracellular degradation or a combination of these two processes. Several lines of evidence suggest that synthesis is altered. First, a detailed characterization of the immunological, physicochemical and kinetic properties of the protein from each line indicates that the polypeptides are identical in structure and that there is only one molecular form expressed as a function of development. This tends to argue against a mutation within the protein coding sequence of the structural gene that could give rise to a less stable protein that would degrade at a differential rate as a function of development. Second, the segregation of activity levels in each line illustrates additive inheritance that is suggestive of altered synthetic rates. In those enzyme systems where such activity level variation is determined by differential rates of enzyme degradation, inheritance patterns are dominant-recessive (GANSCHOW and SCHIMKE 1969; KING and MCDONALD 1983; LAI and SCANDALIOS 1980). Finally, turnover studies using aminotriazole perturbation illustrated that the rate of degradation of catalase in the two lines was similar, while the rates of synthesis vary by twofold in adult flies. Due to technical problems, aminotriazole perturbation is not feasible in the earlier developmental stages, therefore we can only make direct measurements on synthesis rates in adults. However, these results along with the commonality of catalase structure and kinetics suggest that the major process in determining the temporal pattern of catalase expression throughout development is changing rates of enzyme synthesis.

Genetic analysis has placed the site(s) controlling the temporal pattern of catalase synthesis within or near the cytogenetic interval 75D-76A on the left arm of chromosome *3* that has previously been demonstrated to control levels of enzyme activity, CRM and the rate constant for catalase synthesis as a function of gene dosage responses to segmental aneuploidy (LUBINSKY and BEWLEY 1979; NAHMIAS and BEWLEY 1984a). In addition, we have recently recovered a stable deficiency $Df(3L)Cat^{DH104}$ for the cytological region 75B8-75F1 by γ-irradiation mutagenesis that gives a dosage response when in heterozygous combination with the MN18 and RI33 chromosomes (unpublished data). On this basis, it is suggested that region 75D-75F contains the structural gene for Drosophila catalase. The temporal element described in this paper therefore appears to be linked to the Cat^{+} locus. Due to the lack of naturally occurring markers of the structural gene, *i.e.*, electrophoretic alleles, it is difficult to make statements concerning the functional relationship between the Cat^{+} gene and the temporal site, *i.e.*, *cis* or *trans*. Although most temporal genes that map in close proximity to the relevant structural gene act *cis* (PAIGEN 1979), the murine β-glucuronidase temporal locus is a notable exception that acts in *trans* (LUSIS *et al.* 1983). Therefore, additional experiments utiliz-

ing mutagen-induced structural variants of the Cat^+ gene must be conducted to decipher the mode of action of the temporal site in controlling catalase synthesis in Drosophila. These mutagenesis experiments are currently in progress utilizing the recently isolated deficiency $Df(3L)Cat^{DH104}$ that uncovers the Cat^+ structural gene (MACKAY, HOLLAR and BEWLEY 1986).

The relationship between rates of enzyme synthesis and mRNA levels has been examined in relatively few gene-enzyme systems (PAIGEN, LABARCA and WATSON 1979; KOZAK and RATNER 1980; COOK *et al.* 1986). In this study, experiments designed to measure translatable levels of catalase-mRNA in adult Drosophila by *in vitro* translation suggest that the rate of catalase synthesis in lines RI33 and MN18 is correlated with steady state levels of catalase translatable mRNA, although an alternate interpretation could be that the catalase mRNAs from each line differ in their translational efficiency. Therefore, further studies using a Cat-specific hybridization probe will be required before firm conclusions can be made.

The primary translation product of Drosophila catalase has the same apparent size as the mature subunit identified by protein blotting of whole fly extracts and *in vivo* radiolabeling followed by immunoprecipitation. Therefore, Drosophila catalase does not appear to be synthesized as a larger precursor that is subsequently processed during compartmentalization. These results are consistent with data for yeast and rat liver peroxisomal catalase, in which the *in vivo* and *in vitro* translation products are the same (AMMERER *et al.* 1981; YAMADA *et al.* 1982; ROBBI and LAZAROW 1982), but in contrast to plant glyoxysomal catalases that appear as larger-sized precursor forms (YAMAGUCHI, NISHIMURA and AKAZAWA 1984; SKADSEN and SCANDALIOS 1986). The mechanism whereby animal catalase enters peroxisomes is unknown, although it has been suggested that the information directing catalase to peroxisomes might reside within the primary structure of the protein (ROBBI and LAZAROW 1982). This hypothesis can be readily tested in Drosophila by the induction and isolation of structural gene mutations that alter the pattern of catalase compartmentalization. These experiments are in progress.

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