TITLE: Genetic Variation in Resistance to Ionizing Radiation

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INCREMENTAL REQUEST FOR YEAR 2 (1991) AND PROGRESS REPORT FOR YEAR 1 (JANUARY - JULY, 1990)

ABSTRACT

We proposed an investigation of genetically-determined individual differences in sensitivity to ionizing radiation. The model organism is *Drosophila melanogaster*. The gene coding for Cu,Zn superoxide dismutase (SOD) is the target locus, but the effects of variation in other components of the genome that modulate SOD levels are also taken into account. SOD scavenges oxygen radicals generated during exposure to ionizing radiation. It has been shown to protect against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells, whole mice, and *Drosophila*. Two alleles, S and F, are commonly found in natural populations of *D. melanogaster*; in addition we have isolated from a natural population a "null" (CA1) mutant that yields only 3.5% of normal SOD activity. The S, F, and CA1 alleles provide an ideal model system to investigate SOD-dependent radioresistance, because each allele yields different levels of SOD, so that S > F >> CA1.

The role of SOD levels in radioresistance are being investigated in a series of experiments that measure the somatic and germ-line effects of increasing doses of ionizing radiation. During the first seven months of funding we have completed a number of experiments and are proceeding with many others. We have made progress along all the research lines anticipated for the first year of this grant, as summarized in the following pages.

A. Introduction

The very reactive superoxide anion O_2^- is generated during cell respiration as well as during exposure to ionizing radiation. Organisms have evolved different mechanisms to protect against the deleterious effects of reduced oxygen species. The copper-zinc superoxide dismutase (Cu,Zn SOD, referred to hereafter simply as SOD) is a eukaryotic cytoplasmic enzyme that protects the cell by scavenging superoxide radicals and dismutating them to hydrogen

peroxide and molecular oxygen: $2O_2^{\cdot} + 2H^{+} \rightarrow H_2O_2 + O_2$. SOD has been shown to protect against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells, whole mice, and *Drosophila*. The role of SOD in the defense against the toxicity of oxygen has been demonstrated in other ways as well.

We proposed to elucidate further the role of genetic variation in radioresistance by investigating in *Drosophila melanogaster* the response of different SOD genotypes to ionizing radiation. Two alleles, S and F, exist at the SOD locus in natural populations. The allozymes encoded by them differ in specific activity. Moreover, we have a naturally occurring (not obtained by mutagenesis) "null" SOD allele, CA1, which is transcribed and translated, but exhibits only 3.5% of normal SOD levels. We have thus an ideal system to investigate the role of SOD in radioresistance, because we have three alleles in *D. melanogaster* that code for products with a considerable range in SOD activity: S > F >> CA1 ("null"). The F and S alleles are present in all natural populations of *D. melanogaster*, although their frequencies vary from one to another locality. Moreover, the CA1 allele was also obtained from a natural population.

We summarize the results obtained during the first year of this project, that is, since the award started in January 1990 until the time of this writing, in July 1990. We will report the results following the same subheadings (1-6) under D. Experimental Design and Methods of the original proposal, which were anticipated to begin in Year 1.

It should be made clear at the outset that none of the sets of experiments herein reported has been completed: additional replication, treatments, and experiments will be made in every case. This is a "progress" report with respect to every one of the components of the project.

B. Results

1. Strains and Crosses. Using *Drosophila melanogaster* flies collected from a natural population, 54 experimental lines were generated, 18 homozygous for each of the three *Sod* genotypes, F/F, S/S, and N/N. (For simplicity, we use the symbol N for the naturally occurring null allele that we have characterized as CA1). All experiments are made with flies that are the F_1 progenies from crosses between two experimental lines, so as to avoid the effects of inbreeding.

The use of multiple lines of each genotype is necessitated by two related objectives that are essential to this proposal: (1) to insure that effects attributed to SOD are due to this locus rather than to the genetic background; and (2) to evaluate how genetic variation at other loci modulates the effects attributable to the SOD locus.

2. Lethal-Dose-50 for Different SOD Genotypes. Larvae of the same age are collected from each genotype, washed in a 20% sucrose

solution, and placed in small glass Petri dishes for irradiation. Groups of 30 irradiated larvae are placed in separate vials and the adults emerging from the vials are counted. Two experiments are in progress. One tests larvae irradiated during their third instar, i.e., 82-96 hours after hatching: ten replicates have been completed for each of five radiation treatments in addition to the controls. The second experiment tests larvae irradiated during first instar: ten replicates have been completed for just one treatment and their control. The results to date are shown in Table 1 for both experiments.

Table 1. Viability of irradiated larvae with different *Sod* genotype.

Geno-type	Control	Third Instar					First Instar	
		1K	2K	3K	4K	5K	1.5K	% reduction
F/F	29.2	28.8	26.0	25.8	25.1	3.9	25.4	14.0
S/S	28.5	26.5	25.3	22.2	24.3	3.6	23.3	18.2
N/N	28.1	27.7	26.9	23.9	15.5	1.7	17.4	38.1

The dose is given in kilorads. The numbers in the body of the table are the adults emerging out of 30 irradiated larvae, with 10 replicates for each genotype and treatment.

Two results are noteworthy. (1) Differences in radioresistance attributable to SOD are apparent in both experiments. (2) First-instar larvae are much more susceptible than third-instar larvae to irradiation. When third-instar larvae are irradiated, a major decrease in survival rate appears only at doses of 4Kr for the N/N genotype, whereas for first-instar larvae the viability of the N/N genotype is reduced by 38% already at 1.5Kr.

3. Genotype-Dependent Longevity Effects of Irradiation. First-instar larvae are collected and irradiated or kept as controls. Emerging adult flies are collected daily and placed in groups of 30 virgin males of the same age in a vial. The survivors are counted every 5 days and then transferred, without anaesthesia, to fresh vials. Two sets of controls and two sets of 1.5Kr experiments have been completed. Each set consists of 10 replicate

vials each started with 30 flies. The results for one control and one irradiation set are shown in Table 2.

Table 2. Longevity of males irradiated (1.5Kr) as first instar larvae and of their controls.

DAY	CONTROL			IRRADIATED		
	F/F	S/S	N/N	F/F	S/S	N/N
0	30.0	30.0	30.0	30.0	30.0	30.0
5	30.0	29.7	29.3	29.5	29.5	28.8
10	29.5	29.6	27.9	26.3	28.8	24.5
15	29.0	29.4	27.1	24.8	28.3	21.2
20	28.5	29.1	25.6	21.7	26.0	18.2
25	27.9	28.8	23.9	17.9	23.4	15.1
30	26.6	28.2	22.2	16.8	23.0	14.4
35	26.1	27.9	20.6	15.4	22.3	13.9
40	25.8	27.5	19.3	14.2	21.2	12.8
45	24.7	26.0	18.1	12.6	20.9	11.2
50	24.0	24.9	17.5	12.0	18.8	10.5
55	22.4	23.6	17.3	11.4	17.2	9.2
60	21.3	20.4	17.1	10.5	15.3	7.1
65	19.4	17.7	15.6	9.2	13.0	5.1
70	18.0	16.9	15.2	8.6	11.4	3.1
75	16.2	16.0	14.9	7.3	8.7	1.8
80	14.8	13.0	14.2	4.8	6.7	1.1
85	11.3	8.8	10.7	3.2	5.5	.9
90	6.1	7.4	8.0	2.3	3.9	.3
95	4.6	6.0	5.8	1.5	2.8	.2
100	2.7	4.5	3.0	.6	1.4	0
105	2.2	2.6	1.0	.1	.4	•
110	1.4	1.6	0	0	.1	•
115	.6	.4	•	•	0	•
120	.3	0	•	•	•	•
125	.1	•	•	•	•	•
130	0	•	•	•	•	•
% reduction in longevity				25.7	17.3	34.7

The body of the table gives the average number of flies surviving in 10 vials each started with 30 adult males.

All flies of a given genotype in these two sets were derived from the same four lab strains. The results show, as expected, a decrease in longevity in the irradiated cultures and, also as expected, that the radiosensitivity is as predicted by the levels

of SOD activity, S/S > F/F > N/N. It should be, however, noted that different experimental sets started from different lab strains show that he genetic background has a major effect on longevity, which underscores the need to investigate SOD radioprotective effects in different genetic backgrounds. The significance of the longevity studies is, of course, that oxygen radicals have been postulated as major contributors to the aging process.

4. Sterility-Dose-50 for Different Genotypes. We first measured female fertility by placing a virgin female with two males in a vial and replacing each day a cardboard spoon with standard *Drosophila* food. Results for two levels of irradiation and controls are shown in Table 3. Each entry is based on 60 replications and represents the number of eggs collected in three days. Variation between replicates was, however, enormous as can be inferred from the standard deviations shown in the table. There was also considerable variation from day to day in the same culture.

Table 3. Fertility of females irradiated as third-instar larvae.

Genotype	Dose (Kr)		
	0	1	2
FF	202 ± 43	111 ± 11	122 ± 20
SS	172 ± 24	157 ± 29	127 ± 20
NN	183 ± 26	78 ± 18	85 ± 18

The data are averages for 60 females of the number of eggs laid by a single female in days 4, 7, and 10 after emergence.

We have, therefore, performed a series of experiments, first, with different media and culture conditions in order to find a system which yields less variation and, second, to ascertain age-dependent fertility. We found that single females with two males each in a charcoal medium at the bottom of a vial and changing the female and males daily to a new vial yielded the most consistent results. Figure 1 shows the mean fertilities and standard error (N≈80) for females of different genotypes (FS represents heterozygous females with a father F/F and a mother S/S; and the reciprocal for SF). The pattern of age-dependent fertility variation is shown in Figure

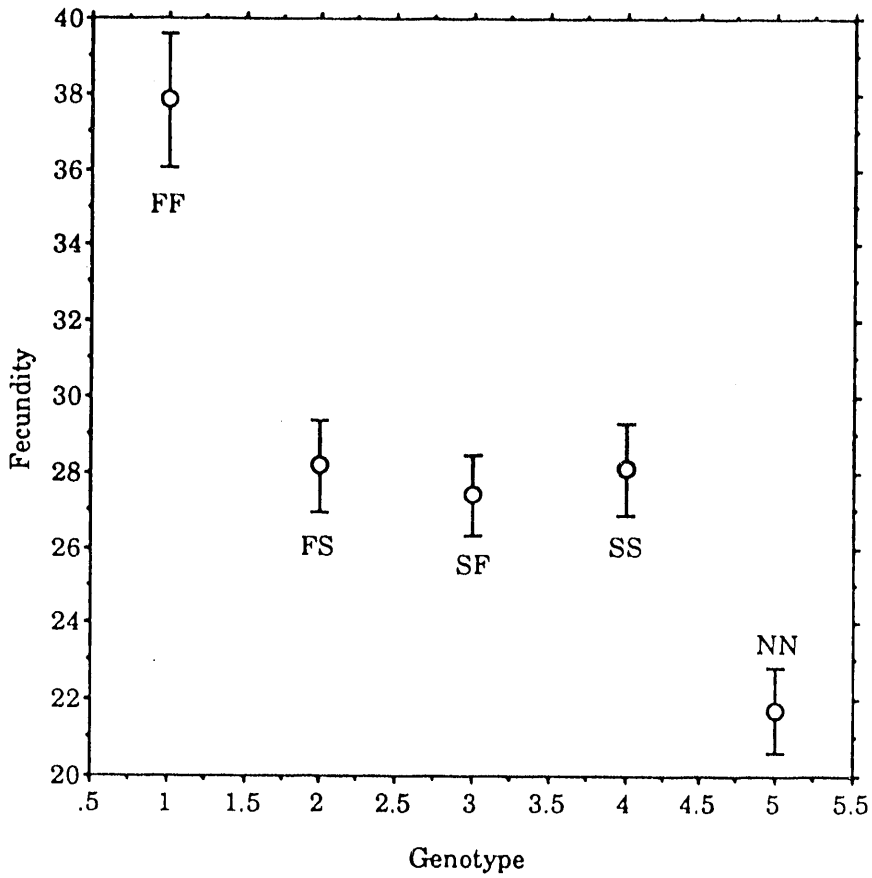

Figure 1. Number of eggs laid in 24 hours for 19-day old females with standard error bars.

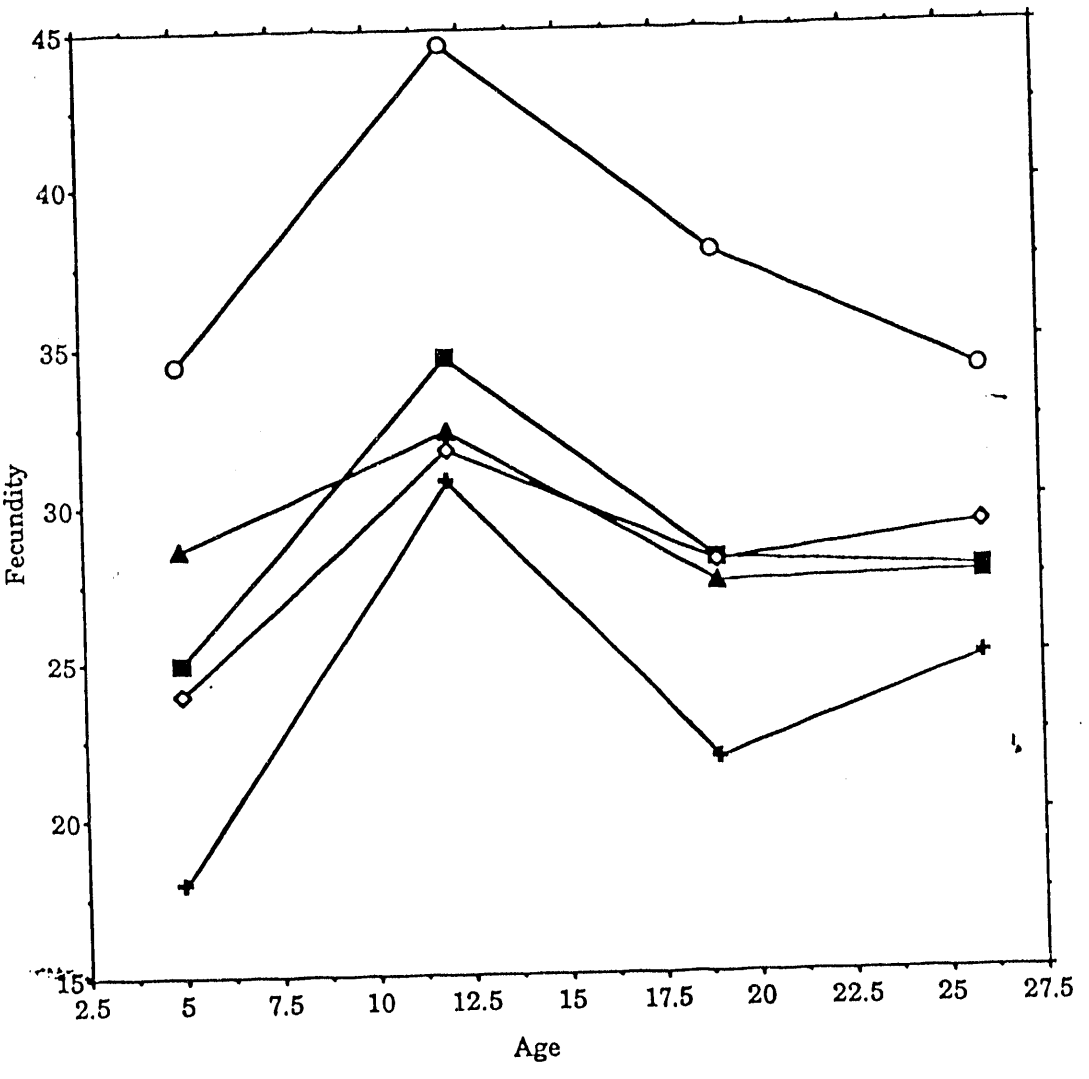

Figure 2.

Mean number of eggs laid in 24 hours for females of different ages and genotypes.

2. We are using the new culture conditions for the experimental treatments.

5. Genotype-Dependent Rate of Lethal Mutations. Immature oocytes (stage-7) are powerful indicators of modification in radiation response. 2-3 days old virgin females of the appropriate genotype are irradiated and then mated with excess males. Eggs are collected from the second 24-hours brood, which represents irradiated immature oocytes at about stage-7. The incidence of sex-linked recessive lethals, X-chromosome losses, and dominant lethality (as indicated by failure of eggs to hatch or to develop to adulthood: "embryonic" and "total") are shown in Table 4. The procedures are as described in the grant application. The results to date do not show significant differences between the S/S and F/F genotypes but a greater radiosensitivity of the N/N at 1.5Kr and 2.5Kr doses.

Table 4. Lethals (%) induced by irradiation of stage 7 immature oocytes with different *Sod* genotypes.

Genotype	Embryonic lethals		Total lethals
	1.5K	2.5K	1.5K
F/F	52.5	70.6	56.6
S/S	53.1	71.2	62.5
N/N	69.4	77.5	78.2

6. Molecular Characterization of the "Null" SOD^{CA1} Allele. This "null" (N) allele was isolated from a natural population. Strains homozygous N/N have enzymatic activity about 3.5% of the F/F homozygotes. The N allele is, therefore, useful for assaying the radioprotective and other physiological roles of SOD because, together with the two commonly occurring alleles, *F* and *S*, provides a three level gradation of enzymatic activity: S/S > F/F >> *N/N*. In *Drosophila* studies, null alleles are typically obtained by mutagenesis, which induces other mutations besides the targeted ones and, hence, handicaps the attribution of particular effects to the mutated locus. Such problem does not occur with our N allele.

We have cloned and obtained the complete sequence of the N allele. As shown in Figures 3 and 4, it carries a 680-bp insertion that starts 47 bp downstream from the start of transcription. The insertion derives from a truncated P element and is identical to

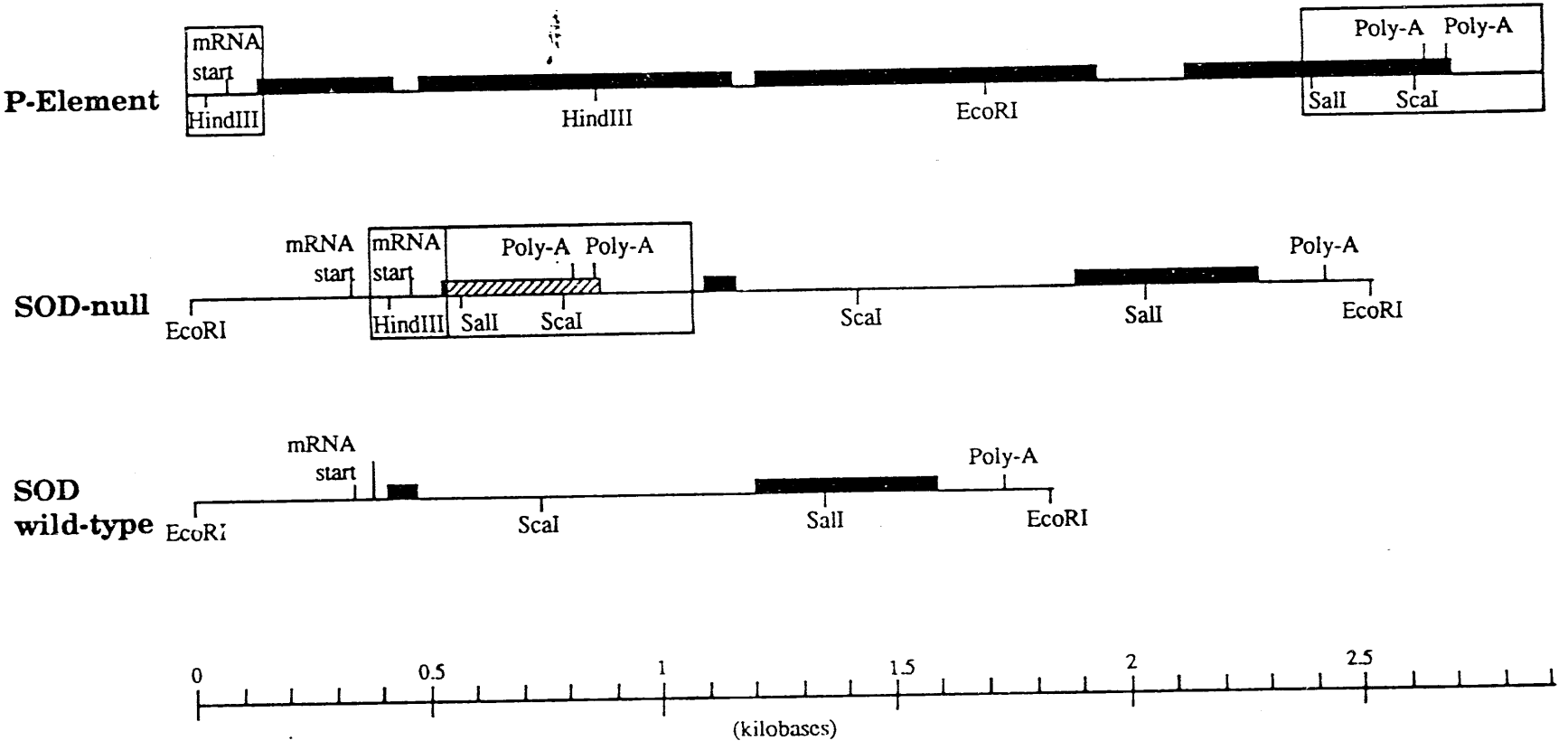

Figure 3. The organization of the Cu, Zn SOD locus in an SOD^{CA1} strain of *D. melanogaster*. Dark boxes represent coding regions. The cross-hatched box is a portion of the 4th P-element ORF. Transcription start points and polyadenylation signals are indicated.

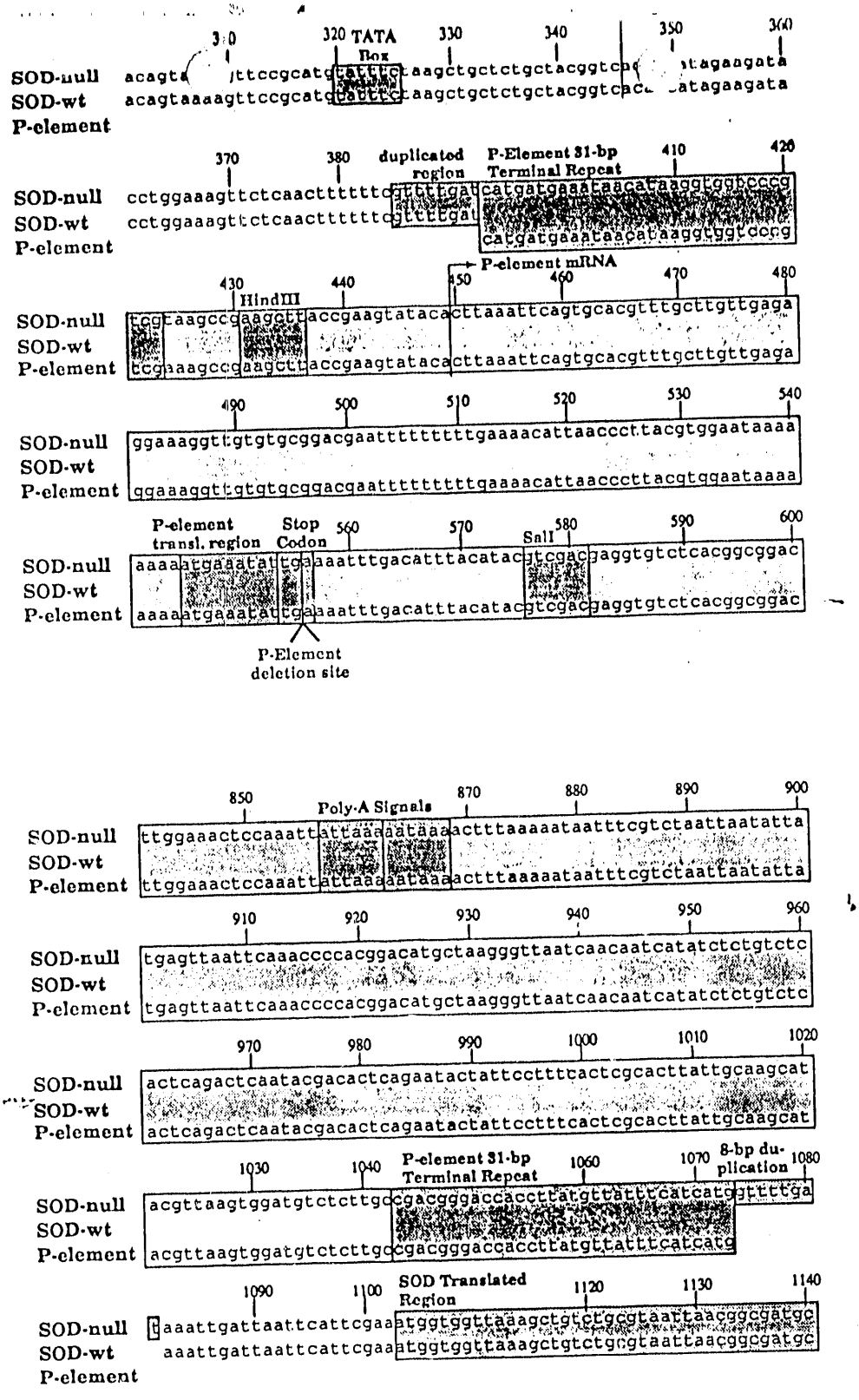

Figure 4. The sequence of the SOD^{CA1} locus adjacent to the insertion of a P-element. The figure shows the alignment of SOD^{CA1}, regions of the inserted P-element and Cu, Zn SOD of *Drosophila melanogaster*. The 8-bp target site duplications as well as the site of the internal deletion within the P-element are indicated.

it, except for one nucleotide substitution at 424. The rest of the sequence is identical to the sequence of an F allele obtained from a Canton-S strain except for a point mutation in the second exon that results in a replacement of Asn by Lys at position 96 of the polypeptide, which confirms that the mature protein encoded by the N allele is the same as S. The deletion in the P element yields a stop codon (see Figure 4). In addition the inserted P-element fragment carries three poly-A signals, two of them adjacent to one another (857 - 868, see Figure 4, which correspond to the second one shown on the top of Figure 3). The diminished expression of *N* is most likely due to a reduction in the rate of transcription, attributable to the insertion of the P element.

C. Concluding Remarks

Seven months after the start of the funding period, we have made substantial progress towards carrying out the research proposed. That SOD plays a role in protecting the cell against ionizing radiation has been conjectured by a number of investigators with various degrees of supporting evidence. The studies we have undertaken seek (1) to confirm the protecting role of SOD against ionizing radiation; (2) to ascertain the magnitude of the effects attributable to the SOD locus; (3) to investigate the germline and somatic cell effects of SOD and the magnitude of the effects for various life-cycle components; and (4) to ascertain the effects attributable to the genetic background. The population genetics approach followed in these studies should be of particular interest to the DOE, precisely because such approach is rarely followed in studies of this kind. Yet, the investigation of SOD effects on germline and somatic cells and various life history components provides invaluable guidance to further investigation of the vulnerable physiological processes protected by SOD. Moreover, the effects of the genetic background must be investigated because genetic variation is important in order to ascertain the effects of ionizing (and other) radiation in natural populations, and because the effects of a particular enzyme or gene locus might be attributable to the genetic context rather than to the locus per se. *Drosophila* provides an ideal model system to investigate some of these issues, which are intractable or extremely costly with mammals and other larger organisms, whereas microorganisms and bacterial systems lack cellular and organ diversity. The genetic manipulations possible with *D. melanogaster* are also of great advantage for the present purposes.

During funding year 2 we will complete several of the experiments in progress and continue others along all the current research lines, according to the timetable proposed in our original application (pp. 19-20).

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