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Note

A *Sod2* Null Mutation Confers Severely Reduced Adult Life Span in Drosophila

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Manuscript received April 14, 2003
Accepted for publication August 26, 2003

ABSTRACT

A null mutation for the *Sod2* gene, *Sod2*^{n283}, was obtained in *Drosophila melanogaster*. Homozygous *Sod2* null (Sod^{n283}/Sod^{n283}) adult flies survive up to 24 hr following eclosion, a phenotype reminiscent of mice, where *Sod2*$^{-/-}$ progeny suffer neonatal lethality. Sod$^{n283/+}$ heterozygotes are sensitive to oxidative stress induced by paraquat treatment.

THE enzyme manganese superoxide dismutase, also known as *Sod2*, detoxifies superoxide radicals (O_2^-) in mitochondria. O_2^- is a byproduct of oxidative phosphorylation in all aerobic organisms. In the case of higher eukaryotes, with relatively more oxygen demand, loss of *Sod2* activity causes pleiotropic phenotypes affecting the heart, brain, muscle, and behavior, culminating in neonatal lethality (reviewed by Wallace 2001). Fundamentally, loss of *Sod2* function causes a net increase in O_2^- load in mitochondria, resulting in mitochondrial membrane damage, which eventually leads to cell death in key tissues like the muscle, heart, and liver (Melov *et al.* 1999; Kokoszka *et al.* 2000). Mitochondrial pathologies in *Sod2* null mice are suggestive of human conditions like Friedrich's ataxia and 3′-hydroxy-3-methyl CoA lyase deficiency (Melov *et al.* 1999). The Drosophila *Sod2* gene has been isolated and characterized (Duttaroy *et al.* 1994, 1997), but no mutant for *Sod2* function has been obtained because of the apparent absence of a deletion straddling the *Sod2* locus (FlyBase 1999). As a first step toward understanding the molecular etiologies associated with the loss of *Sod2* function in specific tissue or cell types, we isolated a null mutant for the *Sod2* gene in Drosophila.

A *P* insertion called KG06854 was identified in the 5′-untranslated region that is located 102 nucleotides upstream from the *Sod2* translation start site in Drosophila (Drosophila *P*-insertion screen/gene disruption project). Adults homozygous for KG06854 are completely viable and fertile. To obtain a null mutant for the *Sod2* gene, we used standard techniques whereby KG06854 was excised using P(Δ2-3), which is the transposase producer (Robertson *et al.* 1988). Putative excision events were selected on the basis of their loss of the white$^+$ phenotype originally associated with KG06854 (Roseman *et al.* 1995). Approximately 300 white-eyed males were selected, and independent lines were established for the purpose of analyzing the *Sod2* gene (Figure 1). Each putative excision line is expected to carry both straight wing (homozygous) and curly wing (heterozygous) flies at a ratio of 1:2; however, in line 283, this ratio was 1:9 (Figure 1). We therefore measured the viability of 283 homozygotes collected within 3 hr of eclosion and monitored their viability every 12 hr. A large majority of these adults (∼98%) died within 24 hr, and by 36 hr all homozygotes had perished (Figure 2). Since *Sod2* knockout mice exhibit neonatal lethality (Li *et al.* 1995; Lebovitz *et al.* 1996) and *Sod2* knockdown in Drosophila reduces its life span (Kirby *et al.* 2002), we investigated the condition of the *Sod2* gene and its expression in line 283. For this purpose an in-gel assay was used that is capable of monitoring the activity of both *Sod1* and *Sod2* proteins, which appeared as two distinct bands (Figure 3). A similar assay with total protein extracted from 283 homozygotes showed no *Sod2* enzyme activity, although their *Sod1* activity remained normal (Figure 3A). Furthermore, 283 heterozygotes (283/Cy), homozygote KG06854, and Canton-S all exhibited perfectly normal *Sod1* and *Sod2* activity (Figure 3A). Even though 283 homozygous flies showed no *Sod2* activity, either an inactive or a truncated *Sod2* protein could still have been present. Western analysis with anti-*Sod2* antibody (Stressgen Biotech, Victoria, BC) failed to show *Sod2* protein in any form in the 283 homozygous flies (Figure 3B). This observation indicates that a *bona fide* null mutation for the *Sod2* gene, called *Sod2*^{n283}, has been obtained in Drosophila.

Could the reduced life-span phenotype of the Drosophila *Sod2*^{n283} allele have resulted from inactivation of *Sod2*, or may some other gene(s) be affected? Because

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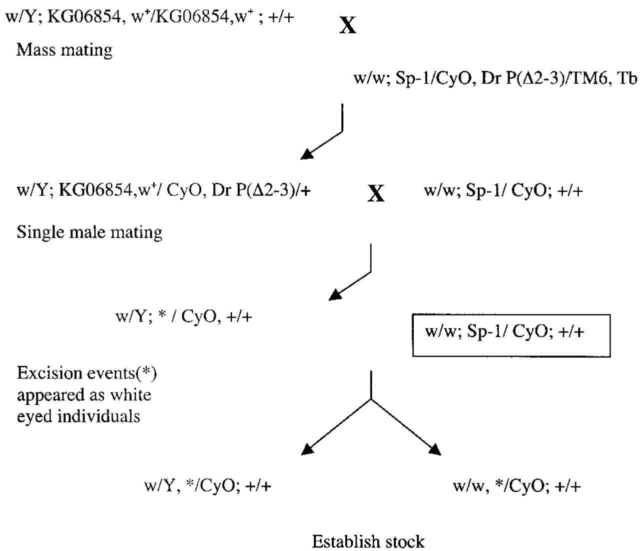

	/ normal wing	*/CyO Curly wing
Expected	1	2
Observed in Line#283	1	9

FIGURE 1.—Schematic of the genetic cross, used for excision of the KG06854 insertion. Putative excision events were analyzed following the establishment of stocks. In line 283, homozygotes for excision chromosomes were recovered far less frequently than their heterozygote sibs.

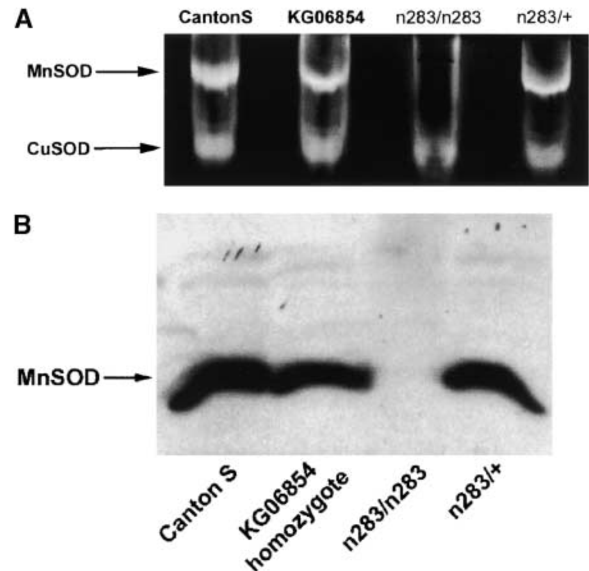

FIGURE 3.—(A) In-gel enzyme assay for MnSOD activity on a 9% nondenaturing discontinuous PAGE gel. Nitro-blue tetrazolium (NBT, 1.23 mM) was used as substrate competing with SOD; 28 mM N',N',N',N'-tetramethylenediamine and 2.8 mM riboflavin were used as nascent free radical donors in a photochemical reaction that converts NBT to blue formazan. A prominent MnSOD band was observed in Canton-S, KG06854 homozygote, and *Sod2*^{n283}/+ but was absent in *Sod2*^{n283}/*Sod2*^{n283}. Cu-Zn SOD was present at similar levels in all the strains. (B) Western blot assay of MnSOD. Total protein extracts were run in a 9% SDS PAGE minigel system and probed with 0.2 μg/ml rabbit anti-MnSOD polyclonal primary antibody (1:5000) and with goat anti-rabbit IgG HRP conjugate secondary antibody (1:50,000; Stressgen Biotech). Signal detection was done using electrochemiluminescence (Amersham Biosciences, Arlington Heights, IL). A 24-kD band specific for MnSOD was observed in wild-type Canton-S, KG06854 homozygote, and *Sod2*^{n283}/+, but was absent in *Sod2*^{n283}/*Sod2*^{n283}.

Sod2^{n283} was recovered through excision, other genes located in the vicinity of KG06854 could be affected. Southern analysis indicated that no genomic sequence other than *Sod2* was affected in *Sod2*^{n283} (Figure 4). *Kpn*I-digested genomic DNA prepared from *Sod2*^{n283}/*Cy* heterozygotes, KG06854 homozygotes, and Canton-S flies showed a 2.0-kb band in the wild-type and *Sod2*^{n283}/*Cy* lanes when *Sod2* DNA was used as a probe (Figure 4). Interestingly, *Sod2*^{n283}/*Cy* heterozygotes picked up an additional band that is 0.53 kb smaller than the 2.0-kb *Kpn*I band. Our analysis revealed that this 1.47-kb band is formed because a large segment of DNA from the *P*-element 3′-end and a portion of the *Sod2* gene are deleted in *Sod2*^{n283}, resulting in the formation of this fusion fragment. Thus, the deletion did not extend beyond the *Sod2* sequence, because the new 1.47-kb band is still recognizable by a *Sod2* probe, so no other gene downstream to *Sod2* is affected in *Sod2*^{n283}. Furthermore, on the 5′-end of the *P* element, a diagnostic PCR band was amplified from *Sod2*^{n283}, KG06854, and Canton-S DNA when all are in homozygous condition (Figure 4). This observation nullifies the possibility that the *P*-element 5′-end is affected in *Sod2*^{n283} during excision. Finally, we confirmed that the reduced longevity pheno-

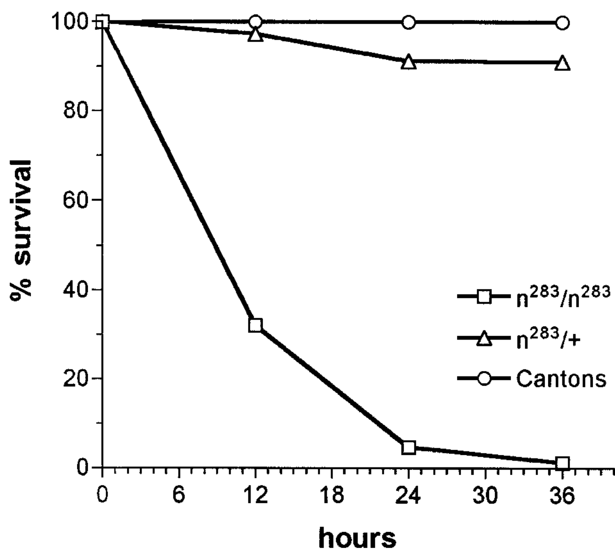

FIGURE 2.—Percentage survivorship among *Sod2*^{n283}/*Sod2*^{n283}, *Sod2*^{n283}/+, and wild-type Canton-S at 25° on normal yeast cornmeal media. A total of 50% of *Sod2*^{n283}/*Sod2*^{n283} flies died within 9 hr, and by 12 hr only 32% survived, compared to the *Sod2*^{n283}/+ or Canton-S, very few of which died during that time.

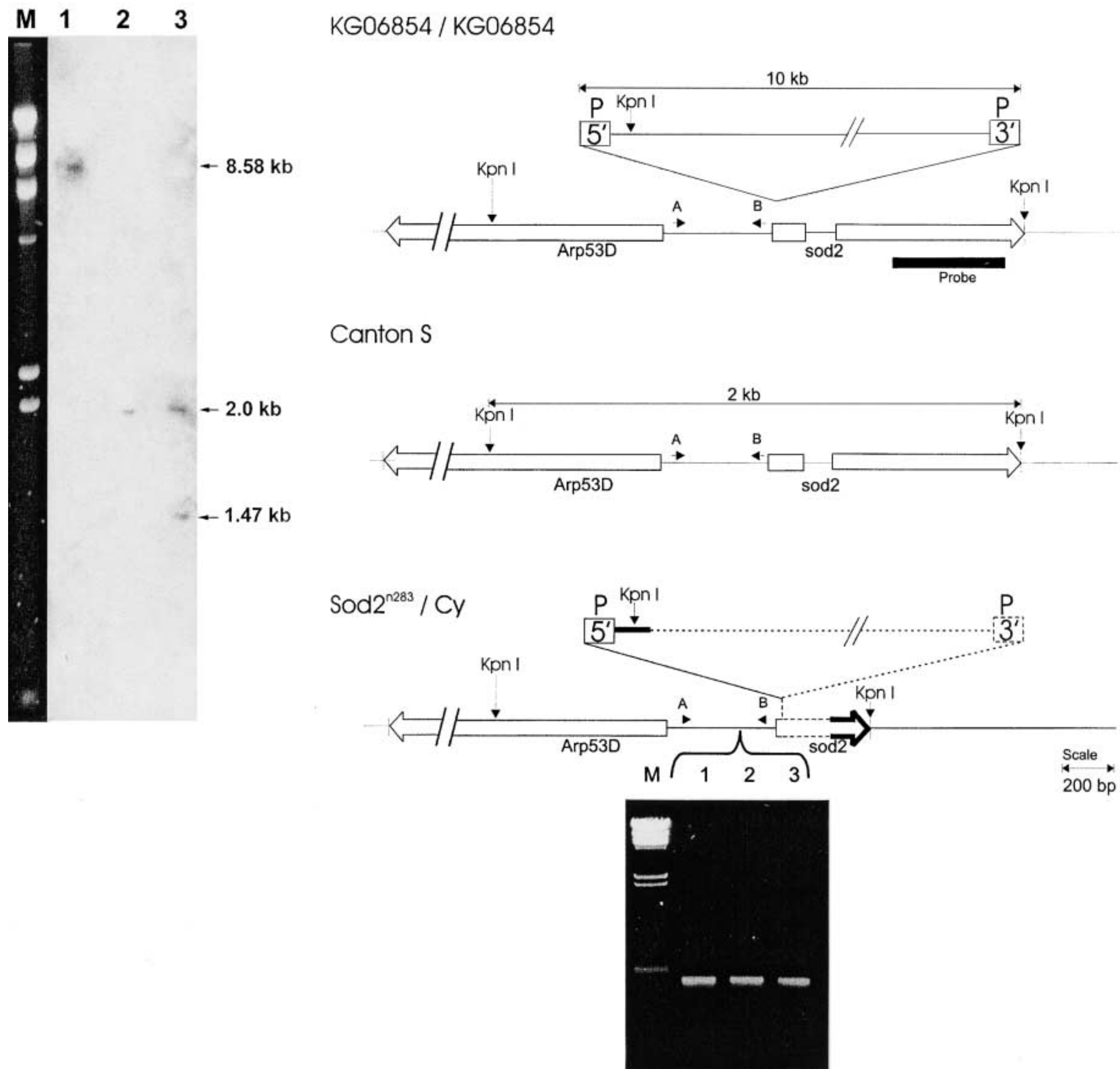

FIGURE 4.—Southern analysis with *Sod2* probe shows a 2.0-kb *Kpn*I band that appears in Canton-S. In *Sod2*^{n283}/ *Cy* heterozygotes, a second band of 1.47 kb appears due to the loss of the majority of the KG06854 element. Since this 1.47-kb excision product is still probable with the *Sod2* probe, it proves that part of the *Sod2* gene is actually affected in *Sod2*^{n283}. Inset shows that the genomic DNA adjacent to the 5′-end of the *P* element remains intact in Sod2^{n283}, since primer A (5′-TGTTTCCAGGAGAGGTTGCT-3′) and B (5′-TCGAAAGACCCCAATCAGTC-3′) amplify a same-size PCR product in homozygous *Sod2*^{n283} (lane 1), KG06458 (lane 2), and Canton-S (lane 3) flies.

type in *Sod2*^{n283} is exclusively due to the loss of *Sod2* function by rescuing this phenotype with an MnSOD transgene that is located on the third chromosome (Mockett *et al.* 1999). More than 97% of *Sod2*^{n283} *vg*/ *Sod2*^{n283} *vg* flies with a recognizable vestigial wing phenotype and carrying the MnSOD transgene on the third chromosome (red eyed) have been found to survive at least 96 hr posteclosion (Table 1). On the other hand, their *Sod2*^{n283} *vg*/*Sod2*^{n283} *vg* sibs without the MnSOD transgene (white eyed) die within 24 hr (Table 1). This led us to conclude that the reduced life-span phenotype of *Sod2*^{n283} resulted from the loss of the *Sod2* sequence.

A Drosophila mutant for Cu-Zn superoxide enzyme, also known as *Sod1*, shows hypersensitivity to oxidative stress condition when *Sod1* homozygotes are fed paraquat (methyl violgen; Phillips *et al.* 1989). Since homozygous *Sod2* flies die so quickly, we tested the susceptibility of *Sod2*^{n283} heterozygotes under oxidative stress conditions by feeding them paraquat. When fed 10 mm paraquat, 50% of *Sod2*^{n283} heterozygotes survived after 24 hr of treatment, whereas 50% of wild-type flies survived for 60 hr (Figure 5). This indicates that *Sod2*^{n283} heterozygotes are approximately twice as sensitive to oxidative stress as wild-type flies.

In summary, we showed that a complete loss of *Sod2* function in Drosophila leads to an extreme reduction

TABLE 1

MnSOD transgene rescues the reduced viability phenotype of $Sod2^{n283}$ homozygotes

w/Y; vg $Sod2^{n283}$/Cy; +/+ × w/w; +/+; [$Sod2^+$ w^+]/[$Sod2^+$ w^+]

↵

w/Y; vg $Sod2^{n283}$/+; [$Sod2^+$ w^+]/+ × w/w; vg $Sod2^{n283}$/Cy; +/+

Genotype	% survival (posteclosion) 0–6 hr	96 hr
w; vg $Sod2^{n283}$/vg $Sod2^{n283}$; [$Sod2^+$ w^+]/+	100	97.5
w; vg $Sod2^{n283}$/vg $Sod2^{n283}$; +/+	100	0

Twenty cohorts of 10 flies/cohort were used.

in life span. This corroborates findings from *Sod2* null mice, which also suffer early lethality. By contrast, multiple pathophysiological conditions were reported due to the loss of *Sod1* function in Drosophila, including reduced life span (Phillips *et al.* 1989), signs of premature aging (Rogina and Helfand 2000), and degeneration (Phillips *et al.* 1995), whereas *Sod1* knockout mice are relatively healthy and show no sign of degeneration (Reaume *et al.* 1996). It is possible that the lack of a third SOD enzyme, the extracellular SOD, makes the insect cells more reliant on *SOD1* activity. The enzyme *Sod2*, active in mitochondria, carries the maximum load of detoxifying superoxide radicals, because ~97% of superoxides are generated in mitochondria. Thus, the

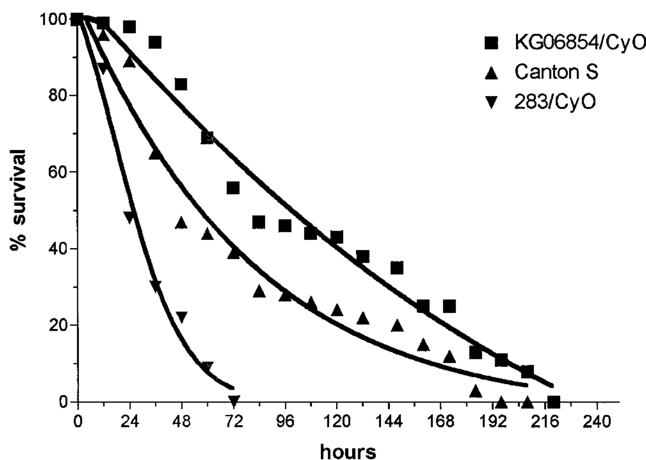

Figure 5.—Survival curve for *$Sod2^{n283}$* heterozygotes and wild-type Canton-S at 25° on 10 mm paraquat and sucrose media. In *$Sod2^{n283}$* heterozygotes, 50% survival was observed after 24 hr compared to 60 hr in Canton-S flies. To compare this effect in a comparative genetic background, we also tested KG06854/Cy flies as a second control. These flies are significantly less sensitive to paraquat treatment compared to *$Sod2^{n283}$/Cy* flies, establishing the point that reduced *Sod2* activity actually made the *Sod^{n283}* heterozygotes more sensitive to oxidative stress.

absence of *Sod2* activity may affect the life span in all organisms, because *Sod2* function is more crucial to aerobic organisms. Extension of the average life span by overexpression of *Sod2* in postmitotic cells (Sun *et al.* 2002) and in motor neurons (J. P. Phillips, T. L. Parkes and A. J. Hilliker, personal communication) further supported this notion. However, these observations are not totally unequivocal, since transgenic overexpression of MnSOD beginning early in development has no beneficial effect on longevity in Drosophila (Mockett *et al.* 1999).

The authors are indebted to William Orr for providing the MnSOD transgenic line. The *P*-element insertion line KG06854 was obtained from the *Drosophila* Stock Center in Bloomington, Indiana. Work was supported by a grant (1R15AG17846-01) from the National Institutes of Health to A.D.

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Communicating editor: J. Birchler