

# Loss-of-Function Mutations in a Glutathione S-Transferase Suppress the *prune-Killer of prune* Lethal Interaction

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## ABSTRACT

The *prune* gene of *Drosophila melanogaster* is predicted to encode a phosphodiesterase. Null alleles of *prune* are viable but cause an eye-color phenotype. The *abnormal wing discs* gene encodes a nucleoside diphosphate kinase. *Killer of prune* is a missense mutation in the *abnormal wing discs* gene. Although it has no phenotype by itself even when homozygous, *Killer of prune* when heterozygous causes lethality in the absence of *prune* gene function. A screen for suppressors of transgenic *Killer of prune* led to the recovery of three mutations, all of which are in the same gene. As heterozygotes these mutations are dominant suppressors of the *prune-Killer of prune* lethal interaction; as homozygotes these mutations cause early larval lethality and the absence of imaginal discs. These alleles are loss-of-function mutations in CG10065, a gene that is predicted to encode a protein with several zinc finger domains and glutathione S-transferase activity.

THE *prune* (*pn*) gene was identified by viable mutations that cause a brownish purple eye-color phenotype (BEADLE and EPHRUSSI 1936). The *pn* gene encodes a single 1.8-kb transcript that is predicted to be translated into a 44.5-kDa protein (FROLOV *et al.* 1994). Nucleotide sequence analysis of *pn* mutants suggested that many of them are null alleles (TIMMONS and SHEARN 1996). Immunoblotting of these same alleles was consistent with this interpretation; no PN protein was detected (TIMMONS and SHEARN 1996). On the basis of sequence similarities PN was predicted to have pyrophosphatase activity (ARAVIND and KOONIN 1998). A recent study of the human ortholog of PN revealed that it has phosphodiesterase activity, specifically cyclic AMP phosphodiesterase activity (D'ANGELO *et al.* 2004). Moreover, there appears to be a correlation between highly metastatic tumors and high levels of PN protein (FORUS *et al.* 2001).

The *abnormal wing discs* (*awd*) gene was identified in a hybrid dysgenic screen for lethal mutations that cause imaginal disc abnormalities (DEAROLF *et al.* 1988a). The *awd* gene encodes a single 0.8-kb transcript (DEAROLF *et al.* 1988b) that is translated into a 17-kDa subunit of a 100-kDa homo-hexameric protein that accounts for 98% of the nucleoside diphosphate kinase activity of larval extracts (BIGGS *et al.* 1990; TIMMONS *et al.* 1995). Nucleoside diphosphate kinases can transfer the terminal phosphate from any nucleoside triphosphate donor to any nucleoside diphosphate acceptor via a ping-pong

mechanism. *Drosophila* AWD is 78% identical to the human NM23A and NM23B proteins that also have nucleoside diphosphate kinase activity (ROSENGARD *et al.* 1989). The *nm23* gene was identified in a screen for genes whose expression is downregulated in metastatic tumor cell lines (STEEG *et al.* 1988). When expressed in *Drosophila*, either NM23A or NM23B produces nucleoside diphosphate kinase activity and can, to varying extents, rescue the *awd* mutant phenotype (XU *et al.* 1996).

*Killer of prune* (*Kpn*) was identified by STURTEVANT (1956) as a nonlethal mutation that causes lethality in flies that lack PN function. LIFSCHYTZ and FALK (1969a) recovered X-ray-induced revertants of *Kpn* that were homozygous lethal. They interpreted these revertants as deletions of one or more vital genes. LIFSCHYTZ and FALK (1969b) also recovered EMS-induced revertants of *Kpn* that were homozygous lethal and allelic to each other. They interpreted these revertants as mutations of a single vital gene. We discovered that this vital gene is *awd* (BIGGS *et al.* 1988). *Kpn* is actually a missense mutation that causes a substitution of Ser for Pro at position 97 of the AWD protein (LASCU *et al.* 1992; TIMMONS *et al.* 1995). We renamed *Kpn* as *awd<sup>Kpn</sup>*.

In hopes of discovering the molecular mechanism of the *pn-awd<sup>Kpn</sup>* lethal interaction we set out to generate mutations that would suppress this interaction and to identify the function of the relevant genes.

## MATERIALS AND METHODS

**Stocks:** Flies were reared on a cornmeal-molasses-agar-yeast extract medium seeded with live yeast at 20°. The *y<sup>1</sup> w<sup>67c</sup>* and *awd<sup>Kpn</sup>* stocks, all of the deficiencies listed in Table 2, most of

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the lethal mutations listed in Table 4, all of the stocks with nonlethal transposon insertions, and the *TM3* balancer chromosome marked with *Kr-GFP* were obtained from the Bloomington Stock Center. The lethal mutations listed in Table 4 that have an "s" in their symbols were obtained from the Szeged Stock Center. The *prune* alleles used ( $pn^{18a}$  and  $pn^{12c}$ ) are null alleles generated in this laboratory on a  $y^1 w^{67c}$  chromosome (TIMMONS and SHEARN 1996). The *TM3* balancer chromosome marked with  $y^+$  was also generated in this laboratory.

**Screen for suppression of endogenous  $awd^{Kpn}$ :** Young male flies (0–24 hr posteclosion) that were hemizygous for  $pn^{18a}$  on the X chromosome and homozygous for *ebony* (*e*) on the third chromosome were fed EMS for 24 hr as described by the SHEARN and GAREN (1974) modification of the procedure initially described by LEWIS and BACHER (1968). After this mutagenesis, the males were mated to females that were homozygous for *yellow* (*y*) on attached X chromosomes and were homozygous for  $awd^{Kpn}$ . All of the male progeny of this cross were expected to die because they were hemizygous for *pn* and heterozygous for  $awd^{Kpn}$  (Table 1) unless a suppressor mutation had been induced.

**Assay for suppressor activity on endogenous and transgenic  $awd^{Kpn}$ :** As controls, females that were homozygous for *pn* on the X chromosomes were mated to males that were homozygous for endogenous  $awd^{Kpn}$  on the third chromosomes or for transgenic  $awd^{Kpn}$  on the second chromosomes. The number of male progeny hemizygous for *pn* and heterozygous for endogenous or transgenic  $awd^{Kpn}$  was compared to the number of female progeny heterozygous for *pn* and heterozygous for endogenous or transgenic  $awd^{Kpn}$ . As experimentals, females that were homozygous for *pn* on the X chromosomes and heterozygous for a suppressor on the third chromosome were mated to males that were homozygous for endogenous  $awd^{Kpn}$  on the third chromosome or for transgenic  $awd^{Kpn}$  on the second chromosome. The number of male progeny hemizygous for *pn*, heterozygous for endogenous or transgenic  $awd^{Kpn}$ , and heterozygous for a suppressor was compared to the number of female progeny heterozygous for *pn*, heterozygous for endogenous or transgenic  $awd^{Kpn}$ , and heterozygous for a suppressor.

**Screen for suppression of transgenic  $awd^{Kpn}$ :** Young male flies (0–24 hr posteclosion) that were hemizygous for *y*, *pn*, and *white* (*w*) on the X chromosome were fed EMS for 24 hr as described above. After this mutagenesis, the males were mated to females that were homozygous for *y* and *w* on attached X chromosomes, were homozygous for transgenic  $awd^{Kpn}$  (marked with  $w^+$ ) on the second chromosome, and were homozygous for *e* and a null allele of *awd* on the third chromosome. The NDP kinase activity supplied by transgenic  $awd^{Kpn}$  is sufficient to rescue the lethality caused by the null *awd* allele. All of the male progeny of this cross were expected to die because they were hemizygous for *pn* and heterozygous for transgenic  $awd^{Kpn}$  (Table 1) unless a suppressor mutation had been induced.

**Deletion mapping of  $Su(Kpn)$ :** Female flies heterozygous for  $Su(Kpn)^1$  were mated to males heterozygous for third chromosome deficiencies. A recovery of <1% of adult flies *trans*-heterozygous for  $Su(Kpn)^1$  and a deficiency relative to their heterozygous sibs were considered a failure to complement.

**Screen for mutations that fail to complement the lethality of  $Su(Kpn)$ :** Young male flies (0–24 hr posteclosion) that were hemizygous for *y* and *w* on the X chromosome and homozygous for  $P[neoFRT82B]$  and  $P[NM88Cw+]$  on the third chromosome were fed EMS for 24 hr as described above. After this mutagenesis, the males were mated at 27° to females that were homozygous for *y* and *w* on the X chromosome and heterozygous for *Glued* (*Gl*) and a dominant temperature-sensitive mutation (*DTS*) and a *TM3* balancer chromosome

marked with *Serrate* (*Ser*) and a  $y^+$  transgene. The  $y^+$  transgene was derived (A. SHEARN, unpublished data) from the  $y^+$  transgene on a second chromosome balancer (TIMMONS *et al.* 1993). Male progeny were mated individually to two to three females that were homozygous for *y* and *w* on the X chromosome and heterozygous for  $Su(Kpn)^1$ . If no progeny *trans*-heterozygous for a mutagenized third chromosome and  $Su(Kpn)^1$  were recovered from an individual cross, then males heterozygous for the mutagenized chromosome, recognizable by the  $w^+$  marker, were retested by mating them to females that were homozygous for *y* and *w* on the X chromosome and heterozygous for  $Su(Kpn)^2$ . If no progeny *trans*-heterozygous for a mutagenized third chromosome and  $Su(Kpn)^2$  were recovered from an individual cross, then males and females heterozygous for the mutagenized chromosome were mated to each other to produce a stock.

**Test for suppression of  $pn$ - $awd^{Kpn}$  lethal phenotype:** Females homozygous for  $y pn^{18a} w^-$  on the X chromosome, heterozygous for transgenic  $\langle w^+ awd^{Kpn} \rangle$  over the  $y^+$  *Cyo* balancer on the second chromosome, and heterozygous for  $Su(Kpn)^1$  over the  $y^+$  *TM3 e Ser* balancer were mated to  $y w^-$  males heterozygous for the putative suppressor alleles,  $Su(Kpn)^x w^+$  FRT balanced over  $w^+ TM3 e Sb KrGFP$ . Males that were  $pn P\{awd^{Kpn}\}$  in combination with either  $Su(Kpn)^1$  or  $Su(Kpn)^x$  alleles were identified on the basis of the presence of the balancer chromosomes. The relative suppression was calculated as the ratio of  $[pn-Su(Kpn)^1 / pn P\{awd^{Kpn}\}-Su(Kpn)^1] / [pn-Su(Kpn)^x / pn P\{awd^{Kpn}\}-Su(Kpn)^x]$ . A G-test for independence was used to test the significance of the difference in the ability to suppress the  $pn$ - $awd^{Kpn}$  lethality between  $Su(Kpn)^1$  and  $Su(Kpn)^x$ . Nonsignificant P-values indicate the ratios of  $Su(Kpn)^1$  to  $Su(Kpn)^x$  are equivalent. Ratios <1 with significant P-values indicate  $Su(Kpn)^x$  is weaker than  $Su(Kpn)^1$ . Ratios >1 with significant P-values indicate  $Su(Kpn)^x$  is stronger than  $Su(Kpn)^1$ . An average of 298 male flies were scored from individual crosses. All crosses were set up twice independently. At least 45 suppressor males were counted in each cross. All crosses were carried out at 25°.

**Lethal excisions of an insertion near  $Su(Kpn)$ :** Males that were hemizygous for *y* and *w* on the X chromosome and homozygous for an insertion in 84C6–8 marked with  $y^+$  and  $w^+$  were mated to females that were homozygous for *y* and *w* on the X chromosome and heterozygous for *Dropped* (*Dr*) and a balancer with a transgene that encodes transposase activity (cross I). Male progeny heterozygous for the insertion and the balancer were mated to females homozygous for *y* and *w* on the X chromosome and heterozygous for a deficiency that uncovers  $Su(Kpn)$  and a *TM3* balancer mutation marked with *Ser* and a  $y^+$  transgene (cross II). Male progeny lacking the  $w^+$  marker of the insertion and the marker of the balancer with transposase activity were considered putative excisions of the insertions. These males were mated to females of the same genotype as their mothers (cross III). If no progeny were recovered that were *trans*-heterozygous for the putative excision and the deficiency, then the excision was deemed to have caused a lethal mutation somewhere within the limits of the deficiency. Male progeny heterozygous for this lethal excision were mated to females homozygous for *y* and *w* on the X chromosome and heterozygous for  $Su(Kpn)^2$  (cross IV). If no progeny were recovered that were *trans*-heterozygous for the lethal excision and for  $Su(Kpn)^2$ , then the excision was deemed to have caused a lethal mutation in  $Su(Kpn)$ .

**Immunoblot analysis of endogenous and transgenic AWD<sup>Kpn</sup>:** To compare the protein levels of endogenous AWD<sup>Kpn</sup> to transgenic AWD<sup>Kpn</sup>, an immunoblot of third instar larvae homozygous for  $awd^{Kpn}$  or  $\langle awd^{Kpn} \rangle$  on an *awd* null background was done. A 15% SDS-PAGE gel was loaded with the equivalent of three larvae per well in 2× SDS sample buffer. Purified AWD

polyclonal antibody previously generated in the laboratory (TIMMONS *et al.* 1995) was used at 1:750 to detect the 15-kDa monomers of AWD<sup>K<sup>pm</sup></sup>. The blot was normalized to  $\beta$ -tubulin (E7: Developmental Studies Hybridoma Bank) at 1:500. HRP-conjugated secondary antibodies were used at 1:5000. The blot was quantitated using NIH Image Software.

**Determination of DNA sequence alterations in *Su(K<sup>pm</sup>)* mutants:** Homozygous larvae were identified as viable, non-fluorescent larvae from the stock  $y w; Su(K<sup>pm</sup>)^x w+FRT/w+TM3 e Sb Kr-GFP$ . Larvae were collected between the first and third instar. Heterozygous larvae were used for samples lethal before the first instar. Twenty to 40 larvae were dounced in buffer A (10 mM Tris-HCl pH 7.5, 60 mM NaCl, 10 mM EDTA pH 8.0, 150 nM spermine, 150 nM spermidine, 5% sucrose). An equal volume of buffer B (1.25% SDS, 300 mM Tris-HCl pH 9.0, 100 mM EDTA pH 8.0, 5% sucrose, 100  $\mu$ g/ml Proteinase K) was added and the lysate was incubated at 37° for 1–2 hr for proteinase K digestion. Following digestion, standard phenol/chloroform/isoamylalcohol extraction and ethanol/NaOAc precipitation recovered genomic DNA. Genomic DNA was PCR amplified using standard conditions with Amplitaq Gold Hotstart Taq (Applied Biosciences, Foster City, CA). Primers covered the four exons of CG10065 in six reactions covering the full gene, including intron/exon splice sites: exon 1 primers, (forward) for 5'-TAA ATC GCC GGC GTT TGC, (reverse) rev 5'-GGC GGG AAA CCA TCT CAT TA; exon 2 set A, for 5'-TAC TGA AGG GAA TAT CCA AGG G, rev 5'-GTC GGG CAG TAT CTC GAT CTT; exon 2 set B, for AGC CACAAT CCA CTC ACG G, rev 5'-GCT CTT GCA ATT GAT GTC CGG AT; exon 2 set C, for 5'-ATA CCA TGC CAA GAA CCG TG, rev 5'-GGT TCG CAA ATA CTC TGC CT; exon 3 primers, for 5'-AAG CTA CAA GCA AAC CGG TC, rev 5'-CCG CTG ATT GAT TAC TGC TC; and exon 4 primers, for 5'-TAC CTC TGC GAC AAC TAC G, rev 5'-CAA TTT GGA AGG CAA AAC AAC CTA. Post-PCR, the product was purified from TAE 1% agarose gel by a QIAGEN (Valencia, CA) gel extraction kit and TA cloned (Invitrogen, San Diego) using standard manufacturer protocol. DNA was purified from three to six colonies by standard miniprep protocol (QIAGEN). Homozygous samples were sent in triplicate and heterozygous samples were sent in quadruplicate for direct sequencing (Johns Hopkins University Genetics Core). Samples were sequenced in both directions using primers included in the TA cloning kit, M13 forward and M13 reverse. Sequence data were aligned using Vector NTI software (Invitrogen).

**Stage of lethality of *trans*-heterozygotes:** Female flies heterozygous for *Su(K<sup>pm</sup>)*<sup>1</sup>, *Su(K<sup>pm</sup>)*<sup>2</sup>, or *Su(K<sup>pm</sup>)*<sup>3</sup> balanced over  $w+TM3 e Sb KrGFP$  were crossed to males of each of the *Su(K<sup>pm</sup>)*<sup>x</sup> alleles balanced over  $w+TM3 e Sb KrGFP$ . Larvae that were *trans*-heterozygous were identified on a fluorescent dissecting microscope as lacking GFP expression. These larvae were moved to a fresh vial and reared in the absence of heterozygous sibling competition at 20°. Those that eclosed as adults were scored for non-Sb bristles to confirm that true *trans*-heterozygotes had been selected.

## RESULTS

**Screen for suppression of endogenous *awd*<sup>K<sup>pm</sup></sup>:** Repeating the work of LIFSCHYTZ and FALK (1969b), we had previously mutagenized *awd*<sup>K<sup>pm</sup></sup> males with EMS and recovered revertants of *pn-awd*<sup>K<sup>pm</sup></sup> lethality. All of the revertants we recovered were either second-site point mutations in the *awd* gene or deletions of the *awd* gene (TIMMONS *et al.* 1995). To try to identify suppressors of

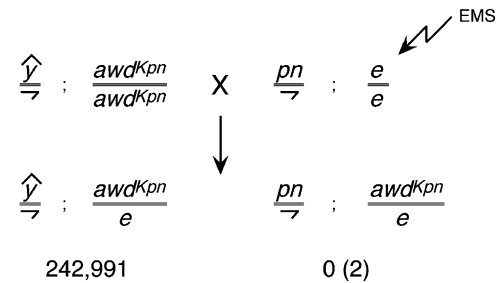


FIGURE 1.—Screen for suppressors of endogenous *awd*<sup>K<sup>pm</sup></sup>. Males were mutagenized with EMS. No suppressors were recovered. The two males recovered were derived by reversion of *awd*<sup>K<sup>pm</sup></sup> in the unmutagenized female parent as described in the text.

*pn-awd*<sup>K<sup>pm</sup></sup> lethality, we used EMS to mutagenize *pn* males and mated them to *awd*<sup>K<sup>pm</sup></sup> homozygous females with attached X chromosomes. All of the male progeny of such crosses would be hemizygous for *pn* and heterozygous for *awd*<sup>K<sup>pm</sup></sup>. We expected that such males would survive only if a suppressor mutation were present. We recovered >240,000 female progeny from this screen and only two males (Figure 1). These males represented putative suppressors. However, even though these putative suppressors were located on the third chromosome they were not on third chromosomes that contained the marker *ebony* (*e*) that was present on mutagenized third chromosomes. Further analysis revealed that these males resulted from second-site mutations of the *awd*<sup>K<sup>pm</sup></sup> gene in the unmutagenized female parents (data not shown). This result demonstrated the extreme selectivity of this screen but indicated that it was unlikely that this protocol would lead to the recovery of suppressor mutations.

**Transgenic *awd*<sup>K<sup>pm</sup></sup> is viable in the absence of *awd* but still kills *prune*:** The AWD protein is normally present at higher levels than are required for viability. Transgenes containing 750 bp of upstream sequence fused to an *awd* cDNA lead to the accumulation of lower levels of AWD than does the endogenous gene, but can nevertheless rescue the lethal phenotype caused by an *awd* null mutation. As examples, two different insertions of such a transgene lead to 42 and 87% viability, respectively, but have 4 and 14%, respectively, as much nucleoside diphosphate kinase activity as wild type (XU *et al.* 1996). This finding led to the idea of modifying our suppressor screen using transgenic rather than endogenous *awd*<sup>K<sup>pm</sup></sup>. We hypothesized that with a lower level of *awd*<sup>K<sup>pm</sup></sup> mutant enzyme, a mutation might be able to suppress the lethal interaction. We constructed an *awd*<sup>K<sup>pm</sup></sup> transgene by mutating the wild-type *awd* transgene described above (TIMMONS and SHEARN 1997). A second chromosome insertion of this *awd*<sup>K<sup>pm</sup></sup> transgene can rescue the lethality caused by an *awd* null mutation (data not shown) but still causes lethality in the absence of *pn* function (Table 1). To compare levels of transgenic and endogenous

TABLE 1

Suppressor activity on endogenous and transgenic *awd<sup>Kpn</sup>*

♀ genotype	No.	♂ genotype	No.
$\frac{pn}{+}; \frac{+}{awd^{Kpn}}$	1804	$\frac{pn}{-}; \frac{+}{awd^{Kpn}}$	0
$\frac{pn}{+}; \frac{Su(Kpn)^1}{+}; \frac{+}{awd^{Kpn}}$	592	$\frac{pn}{-}; \frac{Su(Kpn)^1}{+}; \frac{+}{awd^{Kpn}}$	0
$\frac{pn}{+}; \frac{Su(Kpn)^2}{+}; \frac{+}{awd^{Kpn}}$	305	$\frac{pn}{-}; \frac{Su(Kpn)^2}{+}; \frac{+}{awd^{Kpn}}$	0
$\frac{pn}{+}; \frac{+}{P\{awd^{Kpn}\}}$	687	$\frac{pn}{-}; \frac{+}{P\{awd^{Kpn}\}}$	0
$\frac{pn}{+}; \frac{+}{P\{awd^{Kpn}\}}; \frac{Su(Kpn)^1}{+}$	456	$\frac{pn}{-}; \frac{+}{P\{awd^{Kpn}\}}; \frac{Su(Kpn)^1}{+}$	297**
$\frac{pn}{+}; \frac{+}{P\{awd^{Kpn}\}}; \frac{Su(Kpn)^2}{+}$	398	$\frac{pn}{-}; \frac{+}{P\{awd^{Kpn}\}}; \frac{Su(Kpn)^2}{+}$	386

$P\{awd^{Kpn}\}$ , second chromosome insert of transgene. \*\*Number of males highly significantly different ( $P < 0.01$ ) than number of females according to the *G*-test.

AWD<sup>Kpn</sup> protein, we immunoblotted larval extract using an AWD polyclonal antibody. We found, as expected, endogenous AWD<sup>Kpn</sup> was present at twice the level of transgenic <AWD<sup>Kpn</sup>> (Figure 2).

**Screen for suppression of transgenic *awd<sup>Kpn</sup>*:** To identify suppressors of *pn-awd<sup>Kpn</sup>* lethality, we used EMS to mutagenize *pn* males and mated them to females with attached X chromosomes that were homozygous for transgenic *awd<sup>Kpn</sup>* on the second chromosome and homozygous for an *awd* null mutation on the third chromosome. All of the male progeny of such a cross would be hemizygous for *pn*, heterozygous for transgenic *awd<sup>Kpn</sup>*, and heterozygous for an *awd* null mutation. We expected that such males would survive only if a suppressor mutation were present. We recovered >78,000 female progeny from this screen and four males (Figure 3). One of the males had a second-site mutation in the *pn* gene that reverted the *pn* mutant phenotype (data not shown). The other three males had suppressor mutations on the third chromosome that are allelic. We named the gene that had suffered these mutations *Suppressor of Killer of prune*, *Su(Kpn)*.

***Su(Kpn)* mutations cannot suppress endogenous *awd<sup>Kpn</sup>* but can suppress transgenic *awd<sup>Kpn</sup>*:** The rationale for the modified screen was that putative suppressors might be able to suppress transgenic *awd<sup>Kpn</sup>* because it would produce a lower level of AWD<sup>KPN</sup> protein but not endogenous *awd<sup>Kpn</sup>* because it would produce a higher level of AWD<sup>KPN</sup> protein. We directly tested this

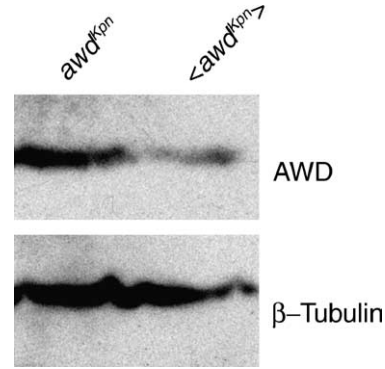


FIGURE 2.—Endogenous AWD<sup>Kpn</sup> protein is present at higher levels than transgenic AWD<sup>Kpn</sup> protein. Lysate from third instar larvae homozygous for endogenous *awd<sup>Kpn</sup>* or homozygous for the transgenic  $P\{awd^{Kpn}\}$  on an *awd* null background were blotted with AWD antibody. A single band at 15 kDa was detected for both samples, representing AWD monomer. The endogenous AWD<sup>Kpn</sup> protein is present at higher levels than the AWD<sup>Kpn</sup> protein. The blot was normalized with  $\beta$ -tubulin to show equal loading of protein.

with the first two of the suppressor alleles recovered (Table 1). We found that neither of these alleles could suppress the lethal interaction between *pn* and endogenous *awd<sup>Kpn</sup>*. Females that were homozygous for *pn* and heterozygous for a suppressor allele were crossed to males that were homozygous for *awd<sup>Kpn</sup>*. Hundreds of female progeny that were heterozygous for *pn*, heterozygous for a suppressor allele, and heterozygous for *awd<sup>Kpn</sup>* were recovered but no males that were hemizygous for *pn*, heterozygous for a suppressor allele, and heterozygous for *awd<sup>Kpn</sup>* were recovered. By contrast, we found that both of these alleles could suppress the lethal interaction between *pn* and transgenic *awd<sup>Kpn</sup>* to varying extents. Females that were homozygous for *pn* and heterozygous for a suppressor allele were crossed to males that were homozygous for transgenic *awd<sup>Kpn</sup>*. Hundreds of female progeny that were heterozygous for *pn*, heterozygous for a suppressor allele, and heterozygous for transgenic *awd<sup>Kpn</sup>* were recovered and hundreds of males that were hemizygous for *pn*, heterozygous for a suppressor allele, and heterozygous for transgenic *awd<sup>Kpn</sup>* were also recovered. The ratio of males to females was 0.65 for *Su(Kpn)<sup>1</sup>* and 0.97 for *Su(Kpn)<sup>2</sup>*.

***Su(Kpn)* cannot suppress *pn*:** The suppressor alleles were recovered in flies that were not only mutant for *pn* but also mutant for *white*. This was done to follow the

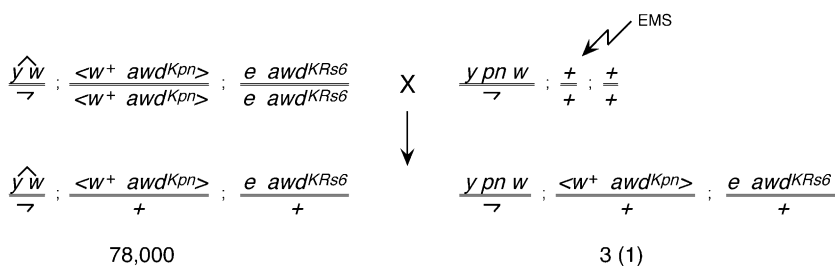


FIGURE 3.—Screen for suppressors of transgenic *awd<sup>Kpn</sup>*. Males were mutagenized with EMS. The four males recovered included three suppressors as well as one *pn* revertant.

**TABLE 2**  
Cytogenetic localization of *Su(Kpn)*

Deficiency (3)	Deleted region	Complementation
<i>th102</i>	72A21; 72D10	+
<i>W10</i>	75A6; 75C2	+
<i>W4</i>	75B10; 75C5	+
<i>H99</i>	75C1; 75C2	+
<i>Cat</i>	75C1; 75F1	+
<i>VW3</i>	76A3; 76B2	+
<i>in61</i>	76F1–3; 77D1–2	+
<i>rdgC-co2</i>	77A1; 77D1	+
<i>ri79c</i>	77B7; 77F5	+
<i>ME107</i>	77F3; 78C8–9	+
<i>31A</i>	78A; 78E	+
<i>Pc-2q</i>	78C5–6; 79A1	+
<i>Delta1AK</i>	79F; 80A	+
<i>2-2</i>	81F4; 83A	+
<i>6-7</i>	82D5; 82F6	+
<i>Tp110</i>	83C1–2; 84B1–2	+
<i>Scr</i>	84A1–2; 84B1–2	+
<i>Antp17</i>	84A5; 84D11–14	–
<i>p712</i>	84D4–6; 85B6	+
<i>XT103</i>	85A2; 85C1–2	+

*awd<sup>Kpn</sup>* transgene that is marked with *w+*. One possibility was that the suppressor acted by suppressing *pn* rather than by suppressing the lethal interaction between *pn* and *awd<sup>Kpn</sup>*. To examine this possibility we generated females *trans*-heterozygous for *pn<sup>18a</sup>* and *pn<sup>12c</sup>*, heterozygous for *white*, and heterozygous for each of the first three suppressor alleles. None of the alleles suppressed the *prune* eye-color phenotype (data not shown). Moreover, as heterozygotes *Su(Kpn)* alleles are not lethal with either endogenous or transgenic *awd<sup>Kpn</sup>*.

**The *Su(Kpn)* gene is in 84C:** All three of the suppressor alleles recovered are homozygous lethal and lethal in all three *trans*-heterozygous combinations. To discover the approximate location of this gene we mapped the lethality by recombination mapping using a previously described protocol (SHEARN and GAREN 1974). The lethality mapped to the middle of the third chromosome between *Gl* and *Sb* (data not shown). To determine the cytogenetic location of *Su(Kpn)*, deficiencies known to remove regions of the chromosome between the *Gl* gene at 70C and the *Sb* gene at 89B were crossed to suppressor alleles and assayed for lack of complementation for the lethal phenotype (Table 2). The failure of *Df(3R)Antp17* to complement indicated that the *Su(Kpn)* gene was located in the region between 84A5 and 84D14. The location was further refined to 84C6–8 by crossing to a second set of deficiencies (Figure 4). The lethality of *Su(Kpn)* is complemented by *Df(3R)Antp<sup>6</sup>* that removes 84B1–C6 and also by *Df(3R)dsx29* that removes 84C8–F6. These data suggest that the cytogenetic location of *Su(Kpn)* is within 84C6–8.

***Su(Kpn)* alleles are loss-of-function mutations:** Our initial expectation was that the suppressor alleles would

be gain-of-function alleles. Finding deficiencies that failed to complement the lethality caused by *Su(Kpn)* alleles allowed us the opportunity to test this expectation. Females that were homozygous for *pn* and heterozygous for *Df(3R)Antp17* were crossed to males that were homozygous for transgenic *awd<sup>Kpn</sup>*. Hundreds of female progeny that were heterozygous for *pn*, heterozygous for the deficiency, and heterozygous for transgenic *awd<sup>Kpn</sup>* were recovered and hundreds of males that were hemizygous for *pn*, heterozygous for the deficiency, and heterozygous for transgenic *awd<sup>Kpn</sup>* were also recovered (data not shown). The fact that the loss of one dose of *Su(Kpn)*+ can provide suppression implies that the suppressor alleles we had recovered were loss-of-function mutations.

***Trans*-heterozygous *Su(Kpn)* mutations cause early larval lethality and an autonomous small disc phenotype:** Homozygous *Su(Kpn)<sup>1</sup>* or *Su(Kpn)<sup>2</sup>* mutants or *trans*-heterozygous *Su(Kpn)<sup>1</sup>/Su(Kpn)<sup>2</sup>* mutants are lethal at the embryo/first larval instar interphase. To examine the imaginal disc phenotype caused by these mutations, anterior halves of *trans*-heterozygous mutant and nonmutant control larvae were cultured in the abdomens of female hosts using a procedure described previously (SIMCOX *et al.* 1989). The anterior halves of larvae continue to develop in host females. When recovered, 10/10 of the nonmutant larvae had normal-appearing imaginal discs (Figure 5A). The anterior halves of mutant larvae also continue to develop in host females. However, when recovered, 0/15 of these mutant larvae had detectable imaginal discs (Figure 5B).

**Additional alleles were recovered in a noncomplementation screen:** The fact that a deficiency can act as a suppressor made it seem possible that our screen for suppressors selected for extreme alleles. To recover a wider range of mutations, we performed a screen for mutations that fail to complement the lethality caused by the original suppressor mutation. The screen was similar in design to ones we have previously reported (SHEARN *et al.* 1978, 1987; SIMCOX *et al.* 1987; MANSFIELD *et al.* 1994). We mutagenized males that had a lethal free isogenic third chromosome marked with a *w+* transgene and mated them to females that were homozygous for *y* and *w* on the X chromosome and were heterozygous for a dominant mutation and a balancer on the third chromosome. Male progeny heterozygous for a mutagenized third chromosome and a balancer chromosome were mated individually to females heterozygous for *Su(Kpn)<sup>1</sup>*. Crosses in which only balancer progeny were recovered represented ones in which the male parent had a putative *Su(Kpn)* mutation. Male progeny were retested by crossing to *Su(Kpn)<sup>2</sup>* heterozygous females (Figure 6). Out of 24,616 single-male crosses, we recovered 16 new alleles.

Because the recovery of alleles from the noncomplementation screen was at a higher frequency than the recovery of suppressors of the *pn-awd<sup>Kpn</sup>* lethal phenotype, we believed the new alleles were of equivalent

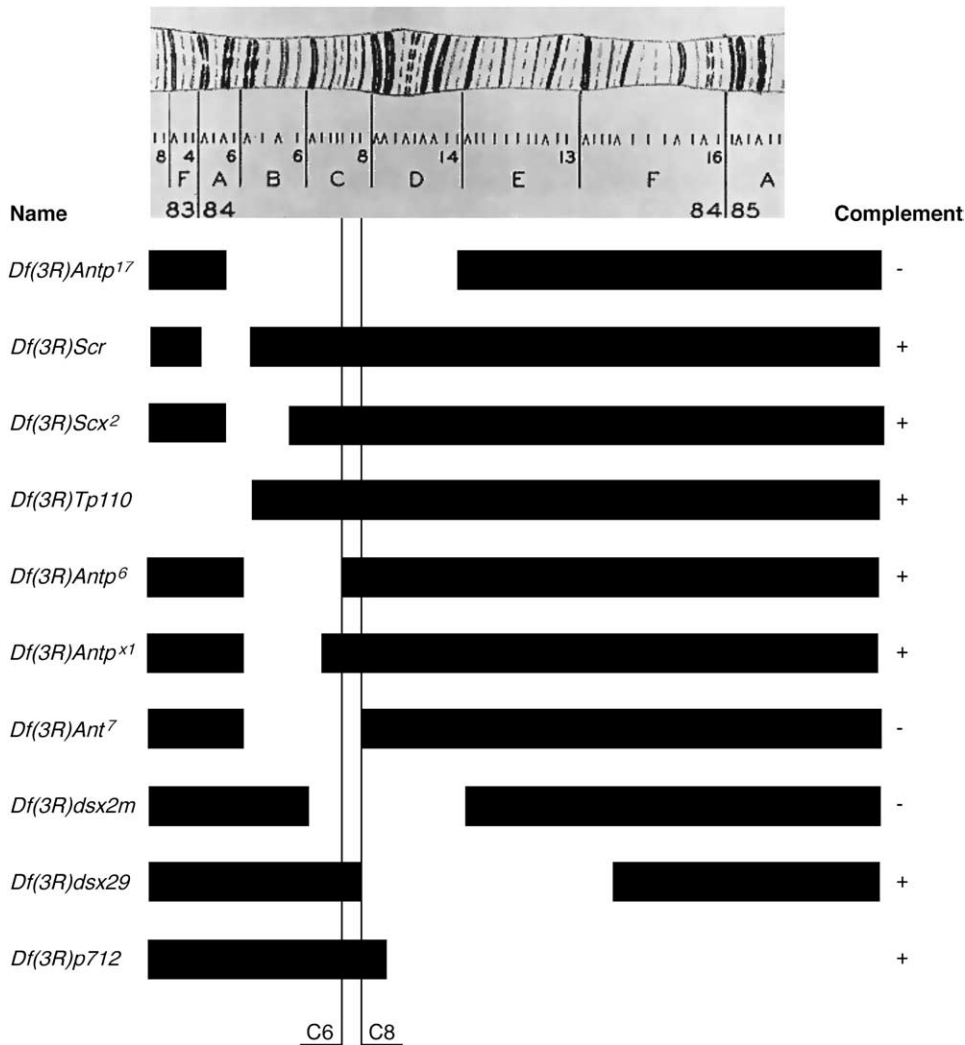


FIGURE 4.—Cytogenetic localization of *Su(Kpn)*. Map of section of right arm of polytene chromosome 3 illustrates the 84 region. Deficiencies lacking specific parts of this region, indicated by gaps in solid bars, were combined with a mutation in *Su(Kpn)*. If this combination was viable it was indicated as “+” in the complement column; if this combination was lethal it was indicated as “-” in the complement column.

strength or weaker than the original *Su(Kpn)* alleles. Further, because these alleles were identified by non-complementation, their ability to suppress the *pn-awd<sup>Kpn</sup>* lethal phenotype was unknown. To test their ability to also suppress the *pn-P{awd<sup>Kpn</sup>}* lethal interaction, males heterozygous for the putative *Su(Kpn)<sup>x</sup>* were crossed to females homozygous for *pn<sup>18a</sup>*, heterozygous for *P{awd<sup>Kpn</sup>}*, and heterozygous for *Su(Kpn)<sup>1</sup>*. Male progeny that were *pn P{awd<sup>Kpn</sup>}* and either *Su(Kpn)<sup>1</sup>* or the putative *Su(Kpn)<sup>x</sup>* from this cross were counted. The strength of the candidate suppressors was compared to that of *Su(Kpn)<sup>1</sup>* and tested for statistical significance using the *G*-test. Our results show that at 25°, 15/16 *Su(Kpn)* alleles suppress the *pn-awd<sup>Kpn</sup>* lethality (Table 3). *AB924* did not suppress the lethality at all. Ten of the alleles were equivalent to *Su(Kpn)<sup>1</sup>* in their ability to suppress, their ratio being equivalent to 1. Six of the alleles were weaker than *Su(Kpn)<sup>1</sup>*. The ratios of the weaker alleles were <1 and statistically significant with a *P*-value <0.005. As previously observed (Table 1), *Su(Kpn)<sup>2</sup>* was stronger than *Su(Kpn)<sup>1</sup>*, having a statistically significant ratio >1.

***Su(Kpn)* fails to complement *l(3)84Cc* but complements all lethal insertion mutations in 84C:** We hoped to use transposon tagging to help identify the DNA sequence that corresponds to the *Su(Kpn)* gene. Toward that end we tested available lethal mutations in 84C, especially lethal transposon insertion mutations for failure to complement *Su(Kpn)* alleles. We found that *Su(Kpn)* alleles fail to complement *l(3)84Cc* that was discovered in a screen for EMS-induced mutations that fail to complement a deletion of the 84B–D region (LEWIS *et al.* 1980; CAVENER *et al.* 1986) but complement every other lethal mutation in the 84B–D region that we tested (Table 4), including all lethal insertion mutations. So, we needed an alternative approach to locate the *Su(Kpn)* DNA sequence.

**Some lethal excisions of *P{EPgy2}EY00044* fail to complement *Su(Kpn)*:** Several nonlethal transposon insertion mutations are available that are located in 84C. We hoped to generate lethal excisions of one of these to help identify the DNA sequence that corresponds to the *Su(Kpn)* gene. To decide which one to excise, we used genetic recombination to find the

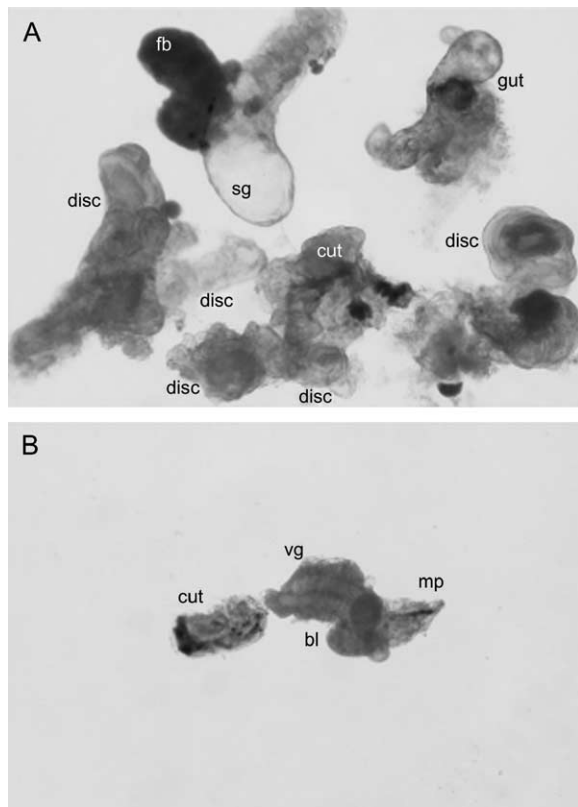


FIGURE 5.—*Su(Kpn)* function is required for growth of imaginal discs. The anterior halves of control and mutant larvae were cultured in the abdomens of host adult females for 3 days. (A) Control halves developed imaginal discs; (B) mutant halves did not develop imaginal discs. Disc, imaginal discs; fb, fat body; sg, salivary gland; cut, larval cuticle; bl, brain lobe; vg, ventral ganglion; mp, mouthparts.

insertion that maps closest to *Su(Kpn)*. We generated females that were *trans*-heterozygous for *Su(Kpn)* and insertions marked with *w*<sup>+</sup> and crossed them to males heterozygous for a deficiency of 84C, *Df(3R)Antp*<sup>7</sup>. One class of recombinant between the insertion and *Su(Kpn)* would generate viable individuals that were *w*<sup>-</sup>. We reasoned that the insertion that gave the lowest frequency of such individuals would be the one closest to *Su(Kpn)*. This process led us to focus our attention on EY00044, a nonlethal EP insertion into 84C6 that is marked with both *y*<sup>+</sup> and *w*<sup>+</sup> (data not shown). We generated excisions of EY00044 that failed to complement *Df(3R)Antp*<sup>7</sup> for lethality and then tested them for failure to complement *Su(Kpn)*<sup>1</sup> (Figure 7). We recovered 88 males that had lethal excisions of EY00044 from a sample of 2769 males (3.2%). Fifty-eight of these excision males were fertile and 12 of them failed to complement *Su(Kpn)*. From this 21% frequency of lethal excisions that fail to complement *Su(Kpn)*, we concluded that *Su(Kpn)* is near to the site of the EY00044 insertion. The EY00044 insertion is within the interval of 2976K and 2977K on the genomic map between CG10061 on the distal side and CG2656 and CG10065

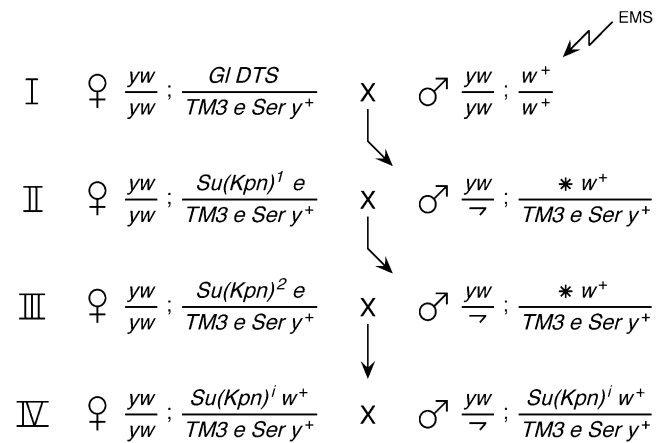


FIGURE 6.—Screen for mutations that fail to complement *Su(Kpn)* mutations. Males were mutagenized with EMS and crossed to balancer females (cross I). Male progeny heterozygous for a mutagenized third chromosome were mated to females heterozygous for *Su(Kpn)*<sup>1</sup> (cross II). If no progeny *trans*-heterozygous for the mutagenized chromosome and *Su(Kpn)*<sup>1</sup> survive, then a putative new allele has been recovered. Male progeny heterozygous for the putative new allele were mated to females heterozygous for *Su(Kpn)*<sup>2</sup> (cross III). If no progeny *trans*-heterozygous for the putative new allele and *Su(Kpn)*<sup>2</sup> survive, then a new allele has been confirmed. *Su(Kpn)*<sup>j</sup> indicates new allele.

on the proximal side (FlyBase). Lethal mutations in CG10061 complement *Su(Kpn)* (Table 4), so we focused our attention on CG2656 and CG10065. CG10065 is within a large intron of CG2656.

**Detection of a broad range of mutations in CG10065 identifies it as the *Su(Kpn)*:** To determine if either CG2656 or CG10065 is the gene identified by *Su(Kpn)* mutations, we used PCR to amplify both genes from mutant larval DNA and determined their nucleotide sequences. Ten *Su(Kpn)* mutants are homozygous lethal in larval stages. These lines were collected as homozygous larvae and analyzed for nucleotide changes in both CG2656 and CG10065. The remaining nine mutant lines are embryonic lethal and were collected as heterozygotes for sequence analysis. PCR and sequencing of homozygous mutant larvae revealed only wild-type sequence in all three exons of CG2656 from eight different alleles (Table 5). However, sequence analysis of the four exons of CG10065 detected mutations in eight homozygous and eight heterozygous larval samples. The nucleotide sequence alterations detected include nine missense mutations [*AB621*, *AB924*, *AP434*, *BX123*, *CL1027*, *CU338*, *DC727*, *DC806*, and *Su(Kpn)*<sup>1</sup>], one small deletion that remains in frame (*BU640*), four premature stop codons [*BW914*, *CK438*, *CX322*, and *Su(Kpn)*<sup>2</sup>], and two deletions that cause a frameshift and premature stop codon [*CZ811* and *Su(Kpn)*<sup>3</sup>]. The presence of nucleotide sequence alterations in CG10065 from nearly every allele examined identifies it as *Su(Kpn)*.

Recently, another group serendipitously studied CG10065 independent of its role in the *pn-awd*<sup>K<sup>pm</sup></sup> lethal

TABLE 3

Suppression of *pn<sup>-</sup>awd<sup>Kpn</sup>* lethality by suppressor alleles

<i>Su(Kpn)<sup>x</sup></i>	<i>N<sup>a</sup></i>	Relative suppression <sup>b</sup>	Significance <sup>c</sup>
<i>AB621</i>	56	0.73	NS
<i>AB924</i>	57	0	<i>P</i> < 0.005
<i>AP320</i>	78	1.25	NS
<i>AP434</i>	141	0.91	NS
<i>AV739</i>	59	0.01	<i>P</i> < 0.005
<i>BS304</i>	73	0.24	<i>P</i> < 0.005
<i>BU640</i>	46	1.4	NS
<i>BW914</i>	114	0.73	NS
<i>BX123</i>	73	0.76	NS
<i>CK438</i>	117	1.12	NS
<i>CL1027</i>	76	0.11	<i>P</i> < 0.005
<i>CU338</i>	102	0.21	<i>P</i> < 0.005
<i>CX322</i>	63	0.83	NS
<i>CZ811</i>	63	1.07	NS
<i>DC727</i>	56	1.39	NS
<i>DC806</i>	57	0.11	<i>P</i> < 0.005
<i>Su(Kpn)<sup>2</sup></i>	98	2.15	<i>P</i> < 0.005
<i>Su(Kpn)<sup>3</sup></i>	52	0.27	<i>P</i> < 0.005

<sup>a</sup> The number of male *pn-awd<sup>Kpn</sup>.Su(Kpn)<sup>1</sup>* progeny recovered. An average of 298 total males were scored per cross.

<sup>b</sup> The ratio of [*pn-Su(Kpn)<sup>1</sup> / pn-awd<sup>Kpn</sup> > -Su(Kpn)<sup>1</sup>*] [*pn-Su(Kpn)<sup>x</sup> / pn-awd<sup>Kpn</sup> > -Su(Kpn)<sup>x</sup>*].

<sup>c</sup> The *P*-value obtained from the *G*-test for independence.

NS denotes nonsignificance, that is, the ratio of *Su(Kpn)<sup>1</sup>:Su(Kpn)<sup>x</sup>* is not statistically different from 1:1. A ratio <1 reflects that *Su(Kpn)<sup>x</sup>* is a weaker suppressor of the *pn-awd<sup>Kpn</sup>* lethality than *Su(Kpn)<sup>1</sup>*. A ratio >1 reflects that *Su(Kpn)<sup>x</sup>* is a stronger suppressor of the *pn-awd<sup>Kpn</sup>* lethality than *Su(Kpn)<sup>1</sup>*.

interaction (DAI *et al.* 2004). Dai *et al.* reported that CG10065 has a single 3.4-kb transcript that encodes a 140-kDa protein. This protein is a novel glutathione S-transferase (GST) containing FLYWCH zinc-finger protein symbolized by the gene name as *gfzf*. Structurally, the protein contains four zinc fingers, an acidic domain, and a GST domain. The mutations we have identified in this gene that we named *Su(Kpn)* occur in both the zinc-finger and GST domains (Figure 8). This suggests that both domains are functionally important in the *Su(Kpn)*-mediated toxicity of the *pn-awd<sup>Kpn</sup>* lethal interaction.

To determine the strength of each mutation with regard to larval viability, we staged larvae *trans*-heterozygous for *Su(Kpn)<sup>1</sup>*, *Su(Kpn)<sup>2</sup>*, or *Su(Kpn)<sup>3</sup>* and for each of the *Su(Kpn)<sup>x</sup>* alleles. We used *trans*-heterozygotes because mutants generated by EMS may give misleading staging information as homozygotes since other mutations may have been simultaneously generated in the genome. Creating *trans*-heterozygous individuals dilutes the second-site mutation effects and allows evaluation of viability, as a result of the *Su(Kpn)* mutations. We conducted the staging experiments at 20° and crossed our original alleles *Su(Kpn)<sup>1</sup>*, *Su(Kpn)<sup>2</sup>*, or *Su(Kpn)<sup>3</sup>* to each of the *Su(Kpn)<sup>x</sup>* alleles. Our results reveal that the *trans*-

heterozygotes lethal at larval stages include missense mutations and all premature stop codons and deletions. Alleles that were viable as *trans*-heterozygous adults were all missense mutations (Figure 8). This suggests that some of the missense mutations are weak mutants that are able to compensate for the loss of function of the extreme alleles and produce viable adult flies.

## DISCUSSION

**Suppressor recovery:** Our screen to recover suppressors of endogenous *awd<sup>Kpn</sup>* did not lead to the recovery of any verified suppressors (Figure 1). We did recover two males, both of which turned out to have revertant mutations in the *awd<sup>Kpn</sup>* gene that was derived from unmutagenized female parents. These revertants provide an estimate of the spontaneous mutation frequency. We recovered 242,991 females. Assuming that a similar number of males would have been recovered but for the *pn:awd<sup>Kpn</sup>* lethality, then that frequency can be estimated to be 2/242,991 or 8/1,000,000 chromosomes tested. The EMS-induced frequency of *awd<sup>Kpn</sup>* revertants we have reported is 65/1,000,000 chromosomes tested (TIMMONS *et al.* 1995). So the induced frequency we recovered is 8.1 times higher than the spontaneous frequency. We had been surprised that 3 of the 16 revertants we recovered in the EMS-induced screen for revertants were deletions (TIMMONS *et al.* 1995). It is possible that one or more of those deletions were actually of spontaneous origin.

Our screen to recover suppressors of transgenic *awd<sup>Kpn</sup>* did lead to the recovery of three verified suppressors. This frequency of recovery, 3/78,000, is significantly different from the lack of recovery of suppressors of endogenous *awd<sup>Kpn</sup>* (0/242,991) at the 5% level according to the *G*-test. This recovery is consistent with the hypothesis that such mutations could not suppress endogenous levels of AWD<sup>KPN</sup> but could suppress transgenic levels of AWD<sup>KPN</sup>. The first two suppressors recovered were used to directly test this hypothesis. Indeed neither *Su(Kpn)<sup>1</sup>* nor *Su(Kpn)<sup>2</sup>* can suppress endogenous *awd<sup>Kpn</sup>* but both can suppress transgenic *awd<sup>Kpn</sup>* (Table 1). The *Su(Kpn)<sup>2</sup>* allele suppresses to a greater extent than does *Su(Kpn)<sup>1</sup>*. We recovered as many males that were hemizygous for *pn*, heterozygous for transgenic *awd<sup>Kpn</sup>*, and heterozygous for *Su(Kpn)<sup>2</sup>* as females that were heterozygous for *pn*, heterozygous for transgenic *awd<sup>Kpn</sup>*, and heterozygous for *Su(Kpn)<sup>2</sup>*. However, we recovered significantly fewer males hemizygous for *pn*, heterozygous for transgenic *awd<sup>Kpn</sup>*, and heterozygous for *Su(Kpn)<sup>1</sup>* than females heterozygous for *pn*, heterozygous for transgenic *awd<sup>Kpn</sup>*, and heterozygous for *Su(Kpn)<sup>1</sup>*. This indicates that *Su(Kpn)<sup>2</sup>* is a stronger suppressor than *Su(Kpn)<sup>1</sup>*. Subsequent sequencing revealed that *Su(Kpn)<sup>2</sup>* causes a nonsense mutation at amino acid 47, whereas *Su(Kpn)<sup>1</sup>* causes a missense mutation at amino acid 169.



**TABLE 4**  
**Complementation tests with lethal mutations in cytogenetic region 84**

Lethal mutation	Gene symbol	Cytogenetic region	Complementation
<i>l(3)s093501</i>		84A3-6	+
<i>l(3)84Bb</i>		84B	+
<i>l(3)84Bc</i>		84B	+
<i>l(3)7A6</i>		84B1-2	+
<i>l(3)L2100</i>		84B2-3	+
<i>l(3)s147406</i>		84B3-6	+
<i>l(3)84Cb</i>		84C	+
<i>l(3)84Ce</i>		84C	+
<i>l(3)s057809</i>		84C	+
<i>l(3)s006313</i>		84C1-2	+
<i>l(3)8C8</i>		84C1-2	+
<i>l(3)02267</i>	<i>Aly</i> = <i>CG1101</i>	84C1-2	+
<i>l(3)S2214</i>	= <i>CG10061</i>	84C4-6	+
<i>l(3)84Cc</i>	<i>gff</i> = <i>CG10065</i>	84C6	-
<i>l(3)s001813</i>		84D	+
<i>l(3)s007403</i>		84D	+
<i>l(3)s023931</i>		84D	+
<i>l(3)s097301</i>		84D	+
<i>l(3)s127405</i>		84D	+
<i>l(3)84Da</i>		84D	+
<i>l(3)84Db</i>		84D	+
<i>l(3)84Dc</i>		84D	+
<i>l(3)84Dd</i>		84D	+
<i>l(3)neo34</i>	<i>lap</i> = <i>CG2520</i>	84D1-14	+
<i>l(3)s127416</i>		84D1-4	+
<i>l(3)s145110</i>		84D1-7	+
<i>l(3)s050115</i>		84D4-8	+
<i>l(3)s002237</i>		84A5; 84D11-14	+
<i>l(3)s015110</i>		84A5; 84D11-14	+
<i>l(3)s091911</i>		84A5; 84D11-14	+
<i>l(3)s026909</i>		84A5; 84D11-14	+
<i>l(3)s026915</i>		84A5; 84D11-14	+
<i>l(3)s036809</i>		84A5; 84D11-14	+
<i>l(3)s042302</i>		84A5; 84D11-14	+
<i>l(3)s044324</i>		84A5; 84D11-14	+
<i>l(3)s051513</i>		84A5; 84D11-14	+
<i>l(3)s113302</i>		84A5; 84D11-14	+
<i>l(3)s118306</i>		84A5; 84D11-14	+
<i>l(3)s139908</i>		84A5; 84D11-14	+
<i>l(3)s140410</i>		84A5; 84D11-14	+

**Allele recovery:** The  $F_2$  screen for *Su(Kpn)* non-complementers yielded, 6.5 alleles per 10,000 chromosomes tested. This is comparable to frequencies recovered in similarly designed noncomplementation screens. For example, in a screen to recover alleles of five different mutations on the third chromosome, we recovered on average 3.6 alleles per 10,000 chromosomes (SHEARN *et al.* 1978). In a screen designed to recover alleles of a different mutation on the third chromosome, we recovered 6 alleles per 10,000 chromosomes (SIMCOX *et al.* 1987). In a screen designed to recover alleles of two other mutations on the third chromosome, we recovered on average 5.2 alleles per 10,000 chromosomes (SHEARN *et al.* 1987). In yet another screen designed to recover alleles of a mutation on the

third chromosome, we recovered on average 5.5 alleles per 10,000 chromosomes (MANSFIELD *et al.* 1994). By contrast, the screen for suppressors of transgenic *awd<sup>Kpn</sup>* yielded only 0.4 alleles per 10,000 chromosomes tested. This much lower frequency suggests that only certain kinds of alleles were recovered in that screen. Since all three alleles recovered in the suppressor screen cause early larval lethality but some alleles recovered in the noncomplementation screen cause later larval lethality, we propose that the suppressor screen selected for extreme alleles. Our analysis of the nucleotide alterations among the various alleles supports this interpretation (Table 5).

**Nucleotide alterations among alleles:** Sequence analysis of mutant *Su(Kpn)* larvae identified a variety of

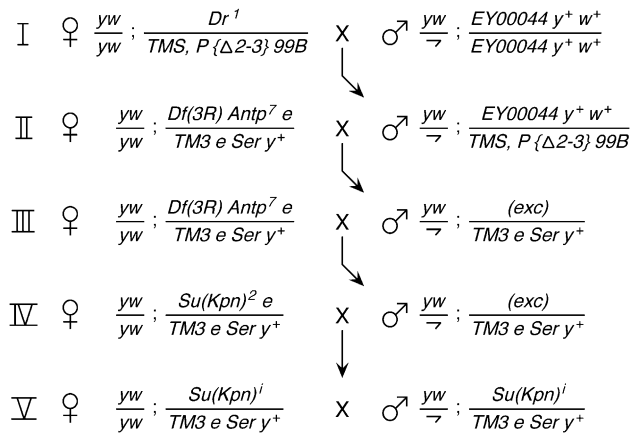
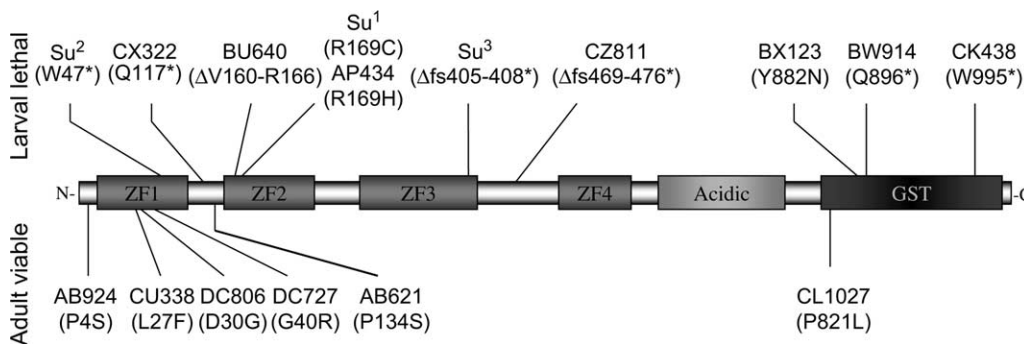


FIGURE 7.—Screen for excision of insertion that maps near to *Su(Kpn)*. Males homozygous for nonlethal insertion were mated to females carrying the source of transposase { $\Delta 2-3$ } (cross I). Males doubly heterozygous for insertion and transposase were mated to females carrying a balancer chromosome (cross II). Male progeny with white eyes represent putative excisions; they were mated individually to deficiency females to assay for the presence of lethal mutation (cross III). Male progeny carrying lethal mutation were mated to females heterozygous to *Su(Kpn)*<sup>2</sup> to test for allelism. *Su(Kpn)*<sup>l</sup> indicates homozygous lethal excision that fails to complement *Su(Kpn)* mutations.

nucleotide alterations in CG10065, ranging from missense mutations to deletions resulting in frameshifts and premature stop codons (Table 5). As we hypothesized from the low recovery rate of mutants from our screen for suppressors of transgenic *awd*<sup>Kpn</sup> (Figure 3), *Su(Kpn)*<sup>l</sup>, *Su(Kpn)*<sup>2</sup>, and *Su(Kpn)*<sup>3</sup> are all severe mutant alleles. We found that both *Su(Kpn)*<sup>2</sup> and *Su(Kpn)*<sup>3</sup> result in premature stop codons. While we have not yet evaluated these mutants for protein accumulation, we believe that *Su(Kpn)*<sup>2</sup> and *Su(Kpn)*<sup>3</sup> will be null mutants; neither one will accumulate any SU(KPN) protein because of the N-terminal stop codon in the mRNA sequence. *Su(Kpn)*<sup>l</sup> is a point mutation in arginine 169. We believe that this residue is essential for proper function of SU(KPN) because another missense muta-



mutations (\*), deletions ( $\Delta$ ), and frameshifts (fs) that introduce nonsense mutations. The stage of lethality of the alleles was evaluated in *trans*—with *Su(Kpn)*<sup>l</sup>, *Su(Kpn)*<sup>2</sup>, and *Su(Kpn)*<sup>3</sup> at 20°. *Trans*-heterozygotes lethal at larval stages are above the diagram, while *trans*-heterozygotes that are viable as adults are below it.

TABLE 5  
Nucleotide alterations in either of two candidate *Su(Kpn)* genes

	CG2656 <sup>a</sup>	CG10065 <sup>b</sup>
Control	WT	WT
AB621	NA	P134S
AB924	WT	P46
AP434	NA	R169H
BU640	WT	$\Delta$ V160–R166
BW914	WT	Q896*
BX123	WT	Y882N
CK438	WT	W995*
CL1027	NA	P821L
CU338	NA	L27F
CX322	NA	Q117*
CZ811	WT	$\Delta$ fsF469–476*
DC727	WT	G40R
DC806	WT	D30G
<i>Su(Kpn)</i> <sup>l</sup>	NA	R169C
<i>Su(Kpn)</i> <sup>2</sup>	NA	W47*
<i>Su(Kpn)</i> <sup>3</sup>	NA	$\Delta$ fsA405–408*

<sup>a</sup> NA, sequence not determined. WT, wild-type sequence.

<sup>b</sup> \*, stop codon.  $\Delta$ , a deletion. fs, frameshift.

tion in this residue was recovered in a second *Su(Kpn)* allele, *AP434*. *AP434* is equivalent to *Su(Kpn)*<sup>l</sup> in its suppression of the *pn-awd*<sup>Kpn</sup> lethal phenotype (Table 3). Further, *AP434* and *Su(Kpn)*<sup>l</sup> are larval lethal in *trans*-heterozygous combinations (Figure 8), making these residues essential for both suppression of the lethal *pn-awd*<sup>Kpn</sup> interaction and viability. *BW914* and *CK438* have C-terminal stop codons in the region that encodes the GST domain. If these mutant proteins accumulate they would lack GST function. They could be extremely valuable for studying the role of GST function in the suppressor activity.

The missense mutations we have detected occur in a wide variety of residues. It is unclear at this time how these residues affect the function of SU(KPN). Because the residues affected in these mutants are found in both

FIGURE 8.—Diagram of the SU(KPN) protein and amino acid changes in the EMS-generated mutants of *Su(Kpn)*. The SU(KPN) contains four zinc-finger domains (ZF1–4), an acidic domain, and a glutathione S-transferase (GST) domain. A broad range of mutations have been identified in both the zinc-finger and the GST domains, including missense mutations, nonsense

the zinc-finger and the GST domains of the protein, we believe that both domains are essential to the function of SU(KPN) *in vivo*. Future structure-function studies will elucidate the role of these residues in SU(KPN) function. Analysis of the *Su(Kpn)* alleles in *trans* with our extreme alleles tested their developmental viability. Interestingly, when these viability data (Figure 8) are compared to their ability to suppress the *pn-awd<sup>Kpn</sup>* lethal interaction (Table 3), it is apparent that there is an uncoupling between survival and suppression. For example, as expected, *AB924*, which is not able to suppress the *pn-awd<sup>Kpn</sup>* lethal interaction, is viable as adults in *trans*-heterozygous combinations with our extreme alleles. However, *Su(Kpn)<sup>3</sup>*, a null allele, is larval lethal at the second instar, but is a rather weak suppressor of the *pn-awd<sup>Kpn</sup>* lethal interaction. It is unclear what the reason for this is at this time. It is possible, although a stop codon exists in the coding region, that there may be read-through and that some protein may be accumulating. Further analysis will be required to determine what protein levels and function of the SU(KPN) are required for the *pn-awd<sup>Kpn</sup>* lethal interaction *vs.* for viability.

In addition to the uncoupling of suppression and viability that emerges from our analysis of the *trans*-heterozygotes, this analysis suggests that the SU(KPN) protein may function as a dimer or multimer. Some alleles, such as *AB621* and *DC727*, that are viable as *trans*-heterozygotes with *Su(Kpn)<sup>1</sup>* are nevertheless strong suppressors as heterozygotes. Because GSTs are known to require dimerization for their function and because zinc fingers can mediate protein-protein interactions (HAYES *et al.* 2005), we hypothesize that the SU(KPN) protein forms multimeric complexes *in vivo* to mediate its effects. Identifying these binding partners will be the subject of future studies.

Although we have not completed analysis of 3 of the 19 alleles recovered in our EMS screens, so far, all of the nucleotide alterations detected have been in the coding region of the SU(KPN). This suggests that protein function, rather than misregulation of gene expression, is responsible for the lethality of the SU(KPN) in the *pn-awd<sup>Kpn</sup>* interaction. Interestingly, we have not yet recovered any mutations in the acidic domain of the protein. The role of the acidic domain is unclear on the basis of its protein sequence and lack of homology to other known domains. One possible reason we have not recovered mutants in this domain is that it is dispensable for SU(KPN) function.

**Protein product of CG10065:** The *Su(Kpn)* gene encodes a novel GST-containing zinc-finger protein (DAI *et al.* 2004). The zinc finger is a very common protein motif, occurring in up to 2% of identified proteins. Zinc-finger proteins have been described as binding both DNA and RNA, as well as mediating protein:protein interactions (RUBIN *et al.* 2000). GSTs are likewise abundant cellular proteins. Thirty-seven

genotype	PN function	AWD function	SU(KPN) function	phenotype
wild-type	XP→X+P	NDP+ATP→NTP+ADP	Y+GSH→YGS	+
<i>pn</i>	XP↑	NDP+ATP→NTP+ADP	Y+GSH→YGS	<i>pn</i> eyes
<i>pn</i> <i>Kpn/+</i>	XP↑	XP+ATP→XPP+ADP NDP+ATP→NTP+ADP	XPP+GSH→XPPGS Y+GSH→YGS	†
<i>pn</i> <i>Kpn/+</i> <i>Su(Kpn)/+</i>	XP↑	XP+ATP→XPP+ADP NDP+ATP→NTP+ADP	Y+GSH→YGS	<i>pn</i> eyes

FIGURE 9.—Model of *pn-awd<sup>Kpn</sup>* lethal interaction. XP is the normal phosphodiester substrate of PN; X and P<sub>i</sub> are the normal products. NDP and ATP are the normal substrates of AWD nucleoside diphosphate kinase; NTP and ADP are the normal products. Y and GSH are the normal substrates of SU(KPN) glutathione S-transferase; Y-GSH is the normal product. Either Y-GSH is required for imaginal disc growth or Y prevents imaginal disc growth. In a *pn* mutant XP accumulates. By itself this is not harmful because XP is not a substrate for normal AWD enzyme. In an *awd<sup>Kpn</sup>* mutant there is slightly less nucleoside diphosphate kinase activity, but this does not cause a phenotype. However, in a *pn-awd<sup>Kpn</sup>* mutant XP accumulates and is phosphorylated by AWD<sup>KPN</sup> to XPP. GSH is added to XPP by SU(KPN) and is converted to XPP-GSH, which is toxic. Loss-of-function mutations in *Su(Kpn)* can suppress the *pn-transgenic awd<sup>Kpn</sup>* lethal interaction because they limit the amount of XPP-GSH that can be produced from the already reduced amount of XPP substrate. Loss-of-function mutations in *Su(Kpn)* cannot suppress the *pn*-endogenous *awd<sup>Kpn</sup>* lethal interaction because the amount of XPP-GSH that can be produced from the abundant amount of XPP substrate is toxic.

GST genes are in *Drosophila*. The majority of GSTs exist as independent proteins that require dimerization to mediate their glutathione-conjugating activity, and it is very rare to find GST domains endogenously fused to other protein domains (DAI *et al.* 2004; UDOMSINPRASERT *et al.* 2004). SU(KPN) is a unique protein because it endogenously fuses a GST domain to multiple zinc-finger domains. Because of the redundancy of GSTs and their overlapping functions, GSTs are able to compensate for each other. We believe SU(KPN) must be unique in its cellular function to have been identified in our EMS screen for suppressors of the *pn-awd<sup>Kpn</sup>* lethal interaction. If SU(KPN) were functioning as a “classic” GST, it would have likely not been detected in our screen due to compensation by other GSTs present in *Drosophila*. Because of the unique fusion of zinc-finger and GST domains in SU(KPN), and because we have identified multiple mutations in both domains, we believe that both the zinc-finger and GST domains are essential for proper function of the SU(KPN). We also know that the wild-type function of SU(KPN) is essential

for the proliferation of imaginal discs, because the alleles *Su(Kpn)<sup>1</sup>*, *Su(Kpn)<sup>2</sup>*, and *Su(Kpn)<sup>3</sup>* recovered in our first screen lack imaginal discs (Figure 5). Future studies will determine the role of zinc-finger and GST domains in the function of SU(KPN) and its role in imaginal disc proliferation.

GSTs are responsible for conjugating reduced glutathione (GSH) to a wide range of native and xenobiotic substrates *in vivo*. Historically, GSTs are considered to be detoxifying enzymes and play a role in the host defense response. However, examples in which GST addition of GSH to substrates creates a product more toxic than the parent substrate exist. An example of this phenomenon is conjugation of GSH with the solvent dichloromethane. This reaction results in the formation of the highly unstable S-chloromethylglutathione, which contains an electrophilic center capable of modifying DNA (HAYES *et al.* 2005). To account for both the *pn-awd<sup>Kpm</sup>* lethal interaction and its suppression by reduced *Su(Kpn)* function we have proposed a hypothetical biochemical model. According to this model, the SU(KPN) conjugation of GSH to a substrate accumulated in *pn-awd<sup>Kpm</sup>* larvae is a toxic event leading to lethality in *pn-awd<sup>Kpm</sup>* flies. Mutations in the *Su(Kpn)* that disrupt the GSH-conjugating ability of SU(KPN) allow *pn-awd<sup>Kpm</sup>* male larvae to survive (Figure 9). Future studies will be directed at identifying the substrate of SU(KPN) and its mechanism in the *pn-awd<sup>Kpm</sup>* lethal interaction.

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