Analysis of the lethal interaction between the *prune* and *Killer of prune* mutations of *Drosophila*

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The third-chromosome mutation *Killer of prune* (*K-pn*) causes no phenotype by itself, but causes lethality in individuals homozygous for the nonlethal X-chromosome mutation *prune* (*pn*). We have recovered 12 gamma-ray-induced revertants of *Killer of prune*. All of the revertants fail to complement a recessive cell lethal mutation in the *abnormal wing discs* (*awd*) gene. We present evidence that *Killer of prune* is a mutation in the *awd* gene. First, revertant *awd^{KR14}* leads to reduced accumulation of the *awd* gene product, but does not affect flanking genes. Second, when a copy of the *awd* gene is cloned from *Killer of prune* homozygous flies and injected into embryos, transformants express the lethal interaction with *prune*. In individuals of the genotype *pn; awd^{K-pn}/awd⁺* the *awd* mRNA is present at normal levels but the *awd* polypeptide fails to accumulate. The absence of the *awd* gene product in such individuals is the cause of death. Although the *awd* polypeptide is a subunit of a cytoplasmic protein, its sequence is similar to subunit V of yeast cytochrome oxidase.

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Killer of prune (*K-pn*) is a conditional dominant lethal mutation of *Drosophila*. It causes lethality only in individuals which are also homozygous or hemizygous for the eye color mutation *prune* (Sturtevant 1956). Otherwise, *K-pn* has no phenotype whether in the heterozygous or homozygous condition (Sturtevant 1956). Since *prune* is sex-linked and *K-pn* is autosomal, all of the male progeny of a cross between homozygous *prune* females and homozygous *K-pn* males are lethal. Lifschytz and Falk (1969) took advantage of this selection system to recover revertants of *K-pn*. They treated *K-pn* males with ethylmethane sulfonate (EMS) or X rays, mated them to *prune* females and recovered the rare male survivors. They found that all of the revertants were homozygous lethal and failed to complement each other. They believed the X-ray-induced revertants represented a set of deletions that overlapped in the region of *K-pn*, so the failure to complement for lethality could have been due to the deletion of vital genes surrounding *K-pn*. They believed that the EMS-induced revertants represented a set of point mutations in a single gene, and the failure to complement for lethality was due to inactivation of that gene. Lifschytz and Falk further suggested that *K-pn* was a neomorphic or antimorphic mutation in this vital gene.

K-pn is located near the tip of the right arm of the third chromosome, close to the location of the *abnormal*

wing discs (*awd*) gene (Dearolf et al. 1988a). The *awd* gene was identified by a lethal mutation, *awd^{b3}*. The wing discs of third-instar mutant larvae are small and have extensive areas of cell death; the brains and ventral ganglia of *awd^{b3}* mutant larvae show extensive vacuolization. The *awd* gene has been isolated by transposon tagging (Dearolf et al. 1988b) and codes for a single mRNA of 0.8 kb. This mRNA is transcribed in wing discs, brains, and ventral ganglia. The *awd^{b3}* mutant gene does not make the normal transcript (Dearolf et al. 1988b). To confirm that it is a null allele, we obtained deletions of the *awd* gene to compare the phenotype of *awd^{b3}* hemizygotes to that of homozygotes. Using the screen for revertants of *K-pn* devised by Lifschytz and Falk (1969), we recovered 12 *K-pn* revertants, all of which were homozygous lethal. We hoped that at least one of the revertants would be a deletion that included the *awd* gene. Unexpectedly, all 12 revertants failed to complement the lethality of *awd^{b3}*. This suggested that *K-pn* and *awd^{b3}* were either two different mutations in the same gene or mutations in adjoining genes. Since we have cloned the *awd* gene, we were able to distinguish between these two possibilities. Our results indicate that *K-pn* is a mutation in the *awd* gene.

Results

Recovery of revertants

From the cross of irradiated *K-pn* males to *prune* (*pn*) females (Fig. 1), we recovered 72,000 females and 35 *pn* males. Twelve of the 35 putative *K-pn* revertants were

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Figure 1. Screen for *Killer of prune* revertants. *K-pn/K-pn* males were irradiated and mated to *pn/pn* females. The only *pn* hemizygous males recovered should be those which carry a revertant of the *K-pn* mutation. All fertile revertants were tested for complementation with the *awd^{b3}* allele and maintained as balanced lethal stocks.

fertile. These revertants are all homozygous lethal. None of the 12 complement the lethality of *awd^{b3}*. Six of the revertants have been examined cytologically. Three have detectable chromosome aberrations in the 100C-D region of the third chromosome. One of the three, *awd^{KR21}*, is a large deletion which includes all of the 100C-D region (Fig. 2). The other three revertants (*awd^{KR7}*, *awd^{KR14}*, and *awd^{KR17}*) have no detectable abnormalities in the 100C-D region.

Figure 2. Salivary gland chromosomes from *awd^{KR21}/+* larvae. The deletion of the 100C-D region is indicated by the arrow.

Mapping the *K-pn* revertants *awd^{KR7}*, *awd^{KR14}*, and *awd^{KR17}*

The three *K-pn* revertants without cytologically detectable abnormalities in the 100C-D region were examined for DNA rearrangements within the *awd* region. The results are shown in Figure 3 and diagrammed in Figure 4. The *awd* gene in both control (*mwh red e*) and *K-pn* flies is contained in a 2.2-kb *Pst*I fragment, while the corresponding *Pst*I fragment from the *TM3* balancer chromosome is 2.4 kb in length (Dearolf et al. 1988b). Because of this polymorphism, changes in the 2.2-kb *Pst*I fragment from the *K-pn* chromosome are detectable in the DNA from heterozygous revertant flies with the genotype *awd^{KR}/TM3*. As shown in Figure 3A (lane 8) and more clearly in Figure 3B (lane 3), the 2.2-kb *Pst*I fragment from the *K-pn* chromosome is missing in *awd^{KR7}*. Otherwise, the DNA fragments generated by restriction enzyme digestion of *awd^{KR7}/TM3* DNA appear identical to those found in *K-pn/TM3* DNA. This lack of novel restriction fragments indicates that the whole region covered by the cloned DNA has been deleted in *awd^{KR7}*.

In *awd^{KR17}*, the 2.2-kb *Pst*I fragment has been replaced by a fragment which is 0.8-kb smaller (Fig. 3A, lane 10). The absence of the 2.2-kb fragment is shown more clearly on a lower-percentage agarose gel (Fig. 3B, lane 5). The 3.4-kb *Sal*I fragment contains the 2.2-kb *Pst*I fragment, and is also reduced by 0.8 kb to 2.6 kb (Fig. 3A, lane 5). Additional mapping data (not shown) indicated that the *Sac*I site close to the 3' end of the *awd* gene was removed by the *awd^{KR17}* deletion. The results localize the 0.8-kb deletion in *awd^{KR17}* toward the 3' end of the *awd* gene, as shown in Figure 4.

The initial whole genome Southern blots of *awd^{KR14}/TM3* DNA revealed no changes in the region of the *awd* gene (Fig. 3A, lanes 4 and 9). Since this revertant was generated by gamma rays and failed to complement the

Figure 3. Analysis of *awd*^{K-pn} revertant alleles *awd*^{KR7}, *awd*^{KR14}, and *awd*^{KR17} by whole genome Southern blots. DNA from adults was digested with *Sal*I (A, lanes 1–5), *Pst*I (A, lanes 5–10; B, lanes 1–5), or *Alu*I (C, lanes 1–6). The digested DNA was fractionated on either a 1.5% agarose gel (A), a 0.8% agarose gel (B), or a 5% polyacrylamide gel (C), and blotted (electroblotted for the 5% polyacrylamide gel) onto a nylon membrane. The membranes were hybridized to a radiolabeled 4.0-kb *Eco*RI–*Sac*I fragment (probe 1, Fig. 4) containing the *awd* gene (A and B), to a radiolabeled *awd* cDNA (C, lanes 1–3), or to a radiolabeled fragment (probe 3, Fig. 4), from the *awd* 5'-flanking sequence (C, lanes 4–6). Genomic DNA was made from adults which were *mwh red e/mwh red e* (A, lanes 1,6; B, lane 1; C, lanes 1,4); *awd*^{K-pn}/*awd*^{K-pn} (A, lanes 2,7; B, lane 2; C, lanes 2,5); *awd*^{KR7}/*TM3* (A, lanes 3,8; B, lane 3); *awd*^{KR14}/*TM3* (A, lanes 4,9; B, lane 4); *awd*^{KR17}/*TM3* (A, lanes 5,10; B, lane 5), or larvae which were *awd*^{KR14}/*awd*^{KR14} (C, lanes 3,6).

lethality of *awd*^{b3}, some change in the *awd* gene must have occurred. To find this change, genomic DNA from *mwh red e*, *K-pn*, and *awd*^{KR14} larvae was digested with *Alu*I, which cuts at the tetranucleotide sequence AGCT. The DNA fragments were hybridized with either an *awd* cDNA or with probe 3 (Fig. 4) which contains 1.0 kb of nontranscribed DNA from the 5' side of *awd*. As shown in Figure 3C (lane 3), there is no detectable difference between the *K-pn* and *awd*^{KR14} revertant DNA when probed with the *awd* cDNA, but when probed with the upstream sequence, two new bands appear in the

awd^{KR14} *Alu*I digest (Fig. 3C, lane 6). This result suggests that a small deletion or an inversion is located in the 5'-nontranscribed region of the *awd* gene in this revertant.

Effect of *K-pn* revertants, *awd*^{KR14} and *awd*^{KR17}, on the accumulation of the *awd* gene transcript

To assess the effect of *awd*^{KR14} and *awd*^{KR17} on the transcription of *awd* and nearby genes, RNA was isolated from *Canton-S*, *awd*^{KR14}/*awd*^{KR14}, and *awd*^{KR17}/*awd*^{KR7} third-instar larvae. The *awd* transcript is abundant at this stage in normal larvae (Dearolf et al. 1988b). The RNA was fractionated on an agarose gel, blotted onto a nylon membrane, then hybridized with a mixture of two cDNAs. One cDNA hybridizes to a 1.35-kb transcript derived from a gene upstream of the *awd* gene; the other hybridizes to the 0.8-kb *awd* transcript. The same filter was then rehybridized with probe 2, which hybridizes to a 0.9-kb transcript from a gene downstream of the *awd* gene (see Fig. 4 for probes utilized). The 1.35-kb transcript is present in all three samples and serves as an indicator of the relative amount of RNA loaded in each lane. The level of the 1.35-kb transcript in *awd*^{KR17}/*awd*^{KR7} is one-half that of the others because *awd*^{KR7} deletes the region which codes for that transcript. We used *awd*^{KR17}/*awd*^{KR7} transheterozygotes in this experiment because a second mutation elsewhere on the *awd*^{KR17} chromosome makes it impossible to get homozygous *awd*^{KR17} third-instar larvae. The 0.8-kb *awd* transcript is present in *Canton-S* RNA, but is missing in both *awd*^{KR14}/*awd*^{KR14} and *awd*^{KR17}/*awd*^{KR7} RNA (Fig. 5A). The 0.9-kb transcript is absent in *awd*^{KR17}/*awd*^{KR7}, but is present at normal levels in *awd*^{KR14}/*awd*^{KR14} (Fig. 5B). The only transcript missing from *awd*^{KR14}/*awd*^{KR14} RNA is the *awd* gene transcript. The fact that reversion of the *K-pn* mutation occurred by eliminating the product of a single gene, the *awd* gene, implies that the *K-pn* mutation is an allele of the *awd* gene.

Germ-line transformation

We have used germ-line transformation to prove that *K-pn* is an allele of *awd*. A genomic library was made by cloning DNA from *K-pn* homozygotes into the EMBL-4 vector (see Materials and methods). A phage containing the *awd* gene was isolated from this library by screening with the *awd* cDNA. A 3.4-kb *Sal*I fragment was isolated from this phage, subcloned into the Carnegie 20 transformation vector, and injected into embryos according to the method of Rubin and Spradling (1982). The Carnegie 20 vector carries a *ry*⁺ gene (Rubin and Spradling 1983). We used *ry*⁺ transformants with insertions on the X or second chromosome to demonstrate that the *K-pn* 3.4-kb *Sal*I fragment rescues the lethality caused by *awd*^{b3} (data not shown). We used a *ry*⁺ transformant with an insertion on the third chromosome to demonstrate that this 3.4-kb *Sal*I fragment is sufficient to cause the lethal interaction with *prune*. The genotype of this transformant is *ry*⁵⁰⁶[*ry*⁺ *awd*^{K-pn}]/*TM3*, *ry*⁵⁰⁶ *e Sb*.

Figure 4. Map of deletions found in *K-pn* revertant DNA. The fine line represents genomic DNA, with restriction sites indicated for *EcoRI* (E), *SalI* (L), *DraI* (D), *ScaI* (C), *PstI* (T), *PvuII* (V), and *SacI* (S). The solid bars represent the *awd* transcript and flanking transcripts. The lower bars indicate the size and position of deletions found in *K-pn* revertant lines *awd*^{KR7}, *awd*^{KR14}, and *awd*^{KR17}. DNA fragments used as probes are shown at the top of the figure. The 3.4-kb *SalI* fragment, also shown at the top of the figure, is the fragment used for germ-line transformation.

Figure 5. Total RNA isolated from third-instar larvae was electrophoresed through a 1.5% agarose formaldehyde gel. The RNA was blotted onto a nylon membrane. Lanes 1–6 correspond to *Canton-S*, *awd*^{KR14}, *awd*^{KR17}/*awd*^{KR7}, *K-pn*, *pn*, and *pn;K-pn/+*, respectively. (A) The filter was hybridized with a mixture of two cDNAs. One, *pc870*, hybridizes to a 1.35 kb transcript; the other, *pc600*, hybridizes to the 0.8-kb *awd* transcript. (B) The filter was washed and hybridized with probe 2 (Fig. 4) which encodes a 0.9-kb transcript.

It contains a single copy of the insert in its genome (J. Biggs, unpubl.). Males from this transformed line were mated to females homozygous for *prune*. No male progeny with the *ry*⁵⁰⁶[*ry*⁺ *awd*^{K-pn}] chromosome were recovered (Fig. 6). This indicates that even in the presence of two copies of the *awd*⁺ gene, one copy of *awd*^{K-pn} is sufficient to cause a lethal interaction with *prune*. The 3.4-kb *SalI* fragment used for transformation contains two coding regions, *awd* and the 1.35-kb transcript upstream of *awd*. However, the fact that *Killer of prune* revertants *awd*^{KR14} and *awd*^{KR17} eliminate the *awd* mRNA without affecting the level of the 1.35-kb mRNA (Fig. 5A) indicates that *Killer of prune* is a mutation in the *awd* gene.

Nucleotide sequence of an *awd* cDNA

To obtain the amino acid sequence of the *awd* gene product, we determined the sequence of the *awd* cDNA, *pc600*. Nucleotides 108–680 in Figure 7 are derived from the cDNA. S1 protection studies (Dearolf et al. 1988b) and primer extension studies (data not shown) indicate the cDNA is full length. Nucleotides 1–107 in Figure 7 were derived from an *awd*^{b3} genomic clone, *pEM2.7a* (Dearolf et al. 1988b).

The proposed cap site begins at nucleotide 105. This sequence, GGCATTC has five of seven nucleotides in common with a reported insect consensus sequence for non-heat shock genes, ATCA(G/T)T(C/T) (Hultmark et al. 1986). The size of the transcript initiating at this site, 565 bp, is consistent with the 570-bp size determined by S1 analysis (Dearolf et al. 1988b). The polyadenylation signal AATAAA (Proudfoot and Brownlee 1976) is located at nucleotide 653, 27 bp from the actual poly(A) sequence. The site of the P-element insertion in *awd*^{b3} begins at nucleotide 164. An intron of approximately 250 bp is close to nucleotide 280. The size of this intron was estimated from the difference in size between

Figure 6. Germ-line transformation of *Drosophila* embryos with *awd*^{K-pn} DNA. The embryos from a cross [P] between *ry/ry* and *ry e awd*^{b3}/*TM3*, *ry Sb e* flies were injected with a DNA fragment containing the *K-pn* allele of *awd*. A 3.4-kb *SalI* fragment from the *K-pn* genome containing the *awd* gene was cloned into the Carnegie 20 transformation vector. This plasmid, along with the 'wings clipped' helper plasmid, was injected according to the method of Rubin and Spradling (1982). Subsequent crosses were carried out as indicated. The various classes of G₂ progeny were examined for the segregation of the *ry*⁺ phenotype to determine the chromosomal location of the insert. Some transformed lines gave G₂ progeny of the *ry e awd*^{b3}/*ry e awd*^{b3} genotype, proving that the *K-pn* allele of *awd* can rescue the *awd*^{b3} null allele; transformed lines which gave *ry*⁺ G₂ progeny of the *ry/TM3*, *ry Sb e* genotype were used for crosses to *pn* females as shown.

cDNA restriction fragments and genomic restriction fragments. Its location was estimated by subtracting the size of the 3' exon (400 bp) from the 3' end of the sequence, 680.

The *awd* sequence (Fig. 7) contains one continuous open reading frame. The codon usage of this reading frame is consistent with that expected for a *Drosophila* gene (O'Connell and Rosbash 1984). In over 90% of eukaryotic genes, translation initiates at the first AUG codon (Kozak 1984). In the *awd* gene, this codon is located at nucleotide 133. According to Kozak (1986), the most favorable context for initiation of translation is the sequence ACCATGG. In *awd* the context of the proposed initiation site is ACAATGG. If this site is used for the start of translation, the *awd* transcript would contain a 28-bp 5'-untranslated leader sequence and would generate a polypeptide of 153 amino acids with a molecular weight of 16–17 kD.

Identification of the *awd* polypeptide

We used antiserum from a rabbit immunized against an *awd*–*trp E* fusion protein to identify the *awd* polypeptide. This antiserum reacts with two polypeptides extracted from wild-type larvae: one of 40 kD and one of 16 kD (Fig. 8). The origin of the 40-kD polypeptide is unclear, but its presence is extremely useful as an in-

ternal standard. Since the size predicted for the *awd* protein based on its cDNA sequence is 16–17 kD, we believe the 16-kD polypeptide is the product of the *awd* gene. This is further supported by the absence of this 16-kD polypeptide in *awd*^{b3} homozygous larvae (Fig. 8).

Although no *awd* transcript is detectable in *awd*^{KR14} larvae (Fig. 5), *awd*^{KR14} does not cause lethality at the stage expected of a null *awd* allele. It causes lethality at the pharate adult stage, whereas the null allele, *awd*^{b3}, causes lethality at the early pupal stage. Since the protein coding region is not affected by *awd*^{KR14}, we considered the possibility that homozygous *awd*^{KR14} larvae survived to a later stage than do homozygous *awd*^{b3} larvae because they accumulate a small amount of *awd* protein. To examine this possibility, we compared the level of *awd* protein in homozygous *awd*^{KR14} larvae to that of control larvae. In homozygous *awd*^{KR14} larvae, the amount of the 40-kD protein is similar to that in control larvae, but the amount of the 16-kD protein is only 20% of that in control larvae (Fig. 9A).

To determine whether or not the *awd* polypeptide exists primarily as a monomer in vivo, we have fractionated an embryo extract on a gel filtration column. Preliminary experiments (J. Biggs, unpubl.) had established that the *awd* polypeptide is present in embryos, that it remains in the supernatant after centrifugation of a homogenate at 12,000g for 15 min, and that the antiserum

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1	AGT GTG ACC ACT TGA CGA TTT CCC ACA ATT CAC CTT GCC GGC ATT TCG	48
49	TAT TCG GGA TTT TTT TGC GGC GAA CAC ATG AAG CTC CTG ATG CTC GGC	96
97	ACA ATT <u>TTG GCA TTC</u> TTT TCT GTA ATC TCG GCG ACA <u>ATG</u> GCG GCT AAC	144
		Met Ala Ala Asn
		P-element
145	AAG GAG AGG ACT TTC ATC ATG GTC AAG CCC GAT GGC GTC CAG CGC GGG	192
	Lys Glu Arg Thr Phe Ile Met Val Lys Pro Asp Gly Val Gln Arg Gly	
193	CTC GTC GGC AAG ATC ATC GAG CGC TTC GAG CAG AAG GGC TTC AAG CTG	240
	Leu Val Gly Lys Ile Ile Glu Arg Phe Glu Gln Lys Gly Phe Lys Leu	
241	GTC GCC CTG AAG TTC ACC TGG GCC TCC AAG GAG CTG CTG GAG AAG CAC	288
	Val Ala Leu Lys Phe Thr Trp Ala Ser Lys Glu Leu Leu Glu Lys His	
289	TAC GCT GAT CTG TCC GCC CGC CCC TTC TTC CCC GGA CTC GTG AAC TAC	336
	Tyr Ala Asp Leu Ser Ala Arg Pro Phe Phe Pro Gly Leu Val Asn Tyr	
337	ATG AAC TCC GGC CCC GTG GTG CCC ATG GTG TGG GAG GGT CTG AAT GTG	384
	Met Asn Ser Gly Pro Val Val Pro Met Val Trp Glu Gly Leu Asn Val	
385	GTC AAG ACC GGT CGC CAG ATG CTC GGC GCC ACC AAC CCC GCC GAC TCG	432
	Val Lys Thr Gly Arg Gln Met Leu Gly Ala Thr Asn Pro Ala Asp Ser	
433	CTG CCC GGC ACC ATC CGC GGT GAC TTC TGC ATT CAG GTC GGA CGC AAC	480
	Leu Pro Gly Thr Ile Arg Gly Asp Phe Cys Ile Gln Val Gly Arg Asn	
481	ATC ATC CAC GGC TCC GAT GCC GTC GAG TCT GCC GAG AAG GAG ATC GCC	528
	Ile Ile His Gly Ser Asp Ala Val Glu Ser Ala Glu Lys Glu Ile Ala	
529	CTG TGG TTC AAC GAA AAG GAG CTG GTC ACC TGG ACC CCG GCC GCC AAG	576
	Leu Trp Phe Asn Glu Lys Glu Leu Val Thr Trp Thr Pro Ala Ala Lys	
577	GAC TGG ATC TAC GAA TAG ACG GCT ACT TTA ACT GTC TGC CCT CGT CTA	624
	Asp Trp Ile Tyr Glu *	
625	AGC TGA ATA CAG ATT GAT TCT TCT AAA <u>GAA ATA AAA</u> TTC CAC AAT AAT	672
		poly-A
673	TAC TAT AT	680

Figure 7. Nucleotide sequence of the *awd* locus. The sequence was determined from the cDNA *pc600* (nucleotides 108–680) and from *pEM 2.7c* (nucleotides 1–171). The predicted 5'-capping site (nucleotides 107–114), the polyadenylation signal (nucleotides 664–669), and the 8 bp flanking the P-element insert in the *awd^{db3}* allele are underlined. The start codon for the 153 amino acid open reading frame is at nucleotide 133.

does not react with the *awd* polypeptide prior to denaturation. The supernatant of an embryo extract was mixed with a set of protein size standards and applied to a Bio-Gel P300 column. One-half of each fraction collected from the column was applied to each of a pair of SDS–polyacrylamide gels. One gel was stained with Coomassie brilliant blue R to determine the elution profile of the size standards. The other gel was used for a Western blot to determine the elution profile of the *awd* polypeptide. The results of this fractionation indicate that the *awd* polypeptide has a molecular weight of 100,000 in the native state (Fig. 10). This suggests the

awd polypeptide is either part of a multisubunit protein complex or part of a multimeric protein.

Similarity of the awd predicted amino acid sequence to a subunit of cytochrome oxidase

A search of the protein database for sequences similar to the predicted *awd* polypeptide revealed the best fit was with a class of small cytochrome oxidase subunits represented by subunit V of the yeast and *Neurospora crassa* enzyme and subunit IV of the bovine heart enzyme. An alignment of the four sequences is shown in Figure 11.

Figure 8. Western blot analysis of the level of *awd* protein in *Canton S* and *awdb³* larvae. Third-instar larvae (approximately 1 larva/10 μ l buffer) were homogenized in SDS sample buffer, the homogenate was boiled, and 40 μ l of each homogenate was loaded into one lane of a 12% SDS–polyacrylamide gel. The total larval proteins were transferred after fractionation onto nitrocellulose, reacted with *awd* immune serum followed by [125 I]protein A (see Materials and methods).

The alignment of the first 50 amino acids of the yeast, bovine, and *Neurospora* cytochrome oxidase subunits is from Powers et al. (1984). In the amino-terminal two-thirds of the polypeptides, 43% of the *awd* amino acids are identical to the amino acids found in either the yeast or the bovine cytochrome oxidase subunit. An additional 14% of the *awd* residues are conservative substitutions. Comparison of the amino-terminal two-thirds of yeast subunit V to bovine subunit IV reveals that 21% of the residues are identical and 11% of the residues are conservative substitutions (Capaldi et al. 1986; Cumsky et al. 1987).

Effect of *prune* and *K-pn* on the accumulation of the *awd* protein

To provide an explanation for the lethal interaction between *prune* and *K-pn*, we have examined the effect of *prune* and *K-pn* on the accumulation of the *awd* transcript and the *awd* polypeptide. The *awd* transcript accumulates to a normal level in larvae homozygous for *prune* or *K-pn* and also in lethal male larvae which result from the cross of *prune* females to *K-pn* males (Fig. 5). The *awd* polypeptide also accumulates to a normal level

Figure 9. Western blot analysis of *awd* protein in mutant larvae. Methods as in Fig. 8. The genotype of the larvae used is indicated at the top of each lane. (A) (WT) *Canton S*; (*awd^{KR-14}*) larvae homozygous for *awd^{KR-14}*. (B) (*awd^{K-pn}*) Larvae homozygous for *awd^{K-pn}*; (*pn*) larvae homozygous for *pn*; (*pn;awd^{K-pn}*) male larvae hemizygous for *pn* and heterozygous for *awd^{K-pn}*. (C) *ash-1^{III-10}* is a late larval lethal included as a control for the effects of larval death.

Figure 10. Bio-Gel P-300 gel filtration chromatography of the *awd* polypeptide. The column was developed as outlined in Materials and methods. Each fraction was run as a lane on an SDS-polyacrylamide gel. The proteins were blotted onto nitrocellulose, treated with antibodies to the *awd* protein followed by [125 I]protein A. The *awd* protein band on the resulting autoradiogram was scanned lane by lane using an ISCO 1312 gel scanner. Gel scanner absorbance units are plotted versus fraction number. Elution fractions of molecular weight standards are indicated.

in *prune* and *K-pn* larvae (Fig. 9B). However, lethal *pn;K-pn/+* larvae accumulate very little *awd* polypeptide (Fig. 9B) despite their accumulation of a normal level of *awd* transcript. This low level of *awd* polypeptide in lethal *pn;K-pn* larvae is not a general property of larval lethality. For example, the level of *awd* polypeptide in an unrelated larval lethal mutant is comparable to wild type (Fig. 9C).

Discussion

Evidence that *K-pn* is a mutation in the *awd* gene

All 12 of the gamma-ray-induced *K-pn* revertants we recovered failed to complement the lethality of *awd^{b3}*. We

examined the nature of three cytologically normal revertants at the molecular level. One of the three, *awd^{KR7}*, is a deletion that removes sequences flanking the *awd* gene in addition to the entire *awd* gene (Fig. 4). Another, *awd^{KR17}*, is missing 800 bp which include the 3'-transcribed region of the *awd* gene and sequences flanking a downstream gene (Fig. 4). The *awd^{KR17}* mutation eliminates the accumulation of both the 0.8-kb *awd* and the 0.9-kb downstream transcripts (Fig. 5).

The most significant of the *K-pn* revertants is *awd^{KR14}* because it only affects the *awd* transcript. No *awd* transcript was detected in third-instar *awd^{KR14}* homozygous larvae (Fig. 5). The *awd^{KR14}* mutation occurred in the 5'-nontranscribed region of the *awd* gene (Figs. 3 and 4).

Figure 11. Sequence alignments between the *awd* protein and cytochrome oxidase subunits. The sequence of beef heart cytochrome oxidase subunit IV is taken from Capaldi et al. (1986). The sequences of the two forms of yeast cytochrome oxidase subunit V are taken from Cumsky et al. (1987). The sequence of the amino terminus of *N. crassa* cytochrome oxidase subunit V is taken from Power et al. (1984). Amino acids that are identical or similar are shaded.

Thus, *awd^{KR14}* reverted *K-pn* and caused a recessive lethal *awd* allele by eliminating a single transcript, the *awd* transcript. We confirmed that *K-pn* is an allele of *awd* by germ-line transformation (Fig. 6). Henceforth, we refer to *K-pn* as *awd^{K-pn}*.

The phenotype of *awd^{KR14}* homozygotes and *awd^{b3}/awd^{KR14}* transheterozygotes is less severe than that of *awd^{b3}* homozygotes. This less severe phenotype suggested that *awd^{KR14}* is not a null allele. Southern and Northern blot analyses showed that *awd^{KR14}* affects an upstream regulatory element of the *awd* gene. Although we were not able to detect accumulation of the *awd* transcript in *awd^{KR14}* homozygous larvae (Fig. 5), we were able to detect a small amount of *awd* polypeptide (Fig. 9A). We believe the reversion of the *K-pn* phenotype that occurred in *awd^{KR14}* to be a consequence of its reduced accumulation of the abnormal *awd^{K-pn}* gene product. The amount of *awd^{K-pn}* polypeptide in *awd^{KR14}/awd^{KR14}* homozygous larvae is adequate to support development until the pharate adult stage, but the amount made in *awd^{KR14}/awd⁺* heterozygous larvae is too low to cause the lethal interaction with *pn*.

Interaction of *pn* with *awd^{K-pn}*

The *awd^{K-pn}* mutation does not alter the level of *awd* RNA (Fig. 5) or *awd* polypeptide (Fig. 8C). Mutations in the *pn* gene also do not alter the level of *awd* RNA (Fig. 5) or *awd* polypeptide (Fig. 9C). Lethal *pn;awd^{K-pn}/awd⁺* larvae also contain the normal level of *awd* RNA (Fig. 5), but they accumulate very little *awd* polypeptide (Fig. 9C). These larvae are heterozygous for a wild-type *awd* allele, so the interaction of *pn* and *awd^{K-pn}* reduces the accumulation of both the *awd^{K-pn}* polypeptide and the *awd⁺* polypeptide. It appears that a certain amount of the *awd^{K-pn}* abnormal polypeptide is required for the lethal interaction with *pn* to occur. Male larvae with the genotype *pn;awd^{K-pn}/awd⁺* would be expected to have a 1 : 1 ratio of mutant to wild-type polypeptide and they die at the beginning of the third-instar larval stage. Male larvae with the genotype *pn;awd^{K-pn} awd⁺/awd⁺* (derived by germ-line transformation; Fig. 6) would be expected to have a 1 : 2 ratio of mutant to wild-type polypeptide and they survive until the end of the third-instar larval stage. Male larvae with the genotype *pn;awd^{KR14}/awd⁺* would be expected to have a 1 : 5 ratio of mutant to wild-type polypeptide, because the *awd^{KR14}* allele produces only 20% as much of the *awd^{K-pn}* polypeptide as does the *awd^{K-pn}* allele (Fig. 9A) and they are viable. These results suggest that for the lethal interaction with *prune* to occur, greater than 17% but as little as 33% of the total *awd* polypeptide must be of the *awd^{K-pn}* form. The evidence from gel-filtration chromatography (Fig. 10) indicates that the 16-kD *awd⁺* polypeptide is part of a 100-kD protein. Perhaps there are two or more *awd* polypeptides in each 100-kD protein, and this protein fails to form or is unstable in a *pn* organism if one or more of those *awd* polypeptides is *awd^{K-pn}*.

The relationship between the *pn* product and the *pn;awd^{K-pn}* interaction

The primary product of the *pn* gene is not yet known. *pn* mutations affect the level of activity of the enzyme GTP cyclohydrolase. This enzyme catalyzes the first step in pteridine biosynthesis and is the product of the *Punch* locus (Mackay and O'Donnell 1983). The eye-color phenotype of *pn* mutations is caused by a reduction in the concentration of red pteridine pigments. The interaction of *pn* with *awd^{K-pn}* is quite specific; *awd^{K-pn}* has no phenotype in combination with other mutations that cause altered accumulations of pteridines (Sturtevant 1956). Using gynandromorphs to do fate mapping, Orevi and Falk (1975) concluded that the lethal interaction has a focus in the ventral region of embryos that gives rise to mesodermal structures and the nervous system. They also found the focus was domineering, i.e., patches of cells that were hemizygous for *pn* and heterozygous for *awd^{K-pn}* and thus genotypically lethal could cause the lethality of other cells that were heterozygous for *pn* and thus genotypically nonlethal. Since *awd* is expressed in the nervous system and in a cell-autonomous fashion (Dearolf et al. 1988a), this suggests that the lethal interaction could be mediated by a diffusible molecule that is released by cells of the nervous system affected by the *pn* mutation (Orevi and Falk 1975).

Similarity of the *awd* amino acid sequence to that of a cytochrome oxidase subunit

The predicted amino acid sequence and observed molecular weight of the *awd* polypeptide is similar to one of the subunits of the mitochondrial enzyme, cytochrome oxidase. However, we do not believe that *awd* is a subunit of cytochrome oxidase. First, in cell fractionation experiments, we can detect the *awd* polypeptide only in the postmitochondrial supernatant, not in mitochondria. Second, the predicted amino acid sequence of the *awd* polypeptide does not contain a signal or leader peptide as do both of the yeast subunit V polypeptides (Cumsky et al. 1987). Third, denatured purified yeast cytochrome oxidase does not cross-react with antiserum raised against an *awd* fusion protein (J. Biggs, unpubl.). Fourth, despite the considerable similarity of the *awd* sequence to that of the yeast and bovine cytochrome oxidase subunits, plots of their relative hydropathicity (Kyte and Doolittle 1982) indicate that *awd* has a different pattern than do the cytochrome oxidase subunits, which have patterns strikingly similar to each other (data not shown). Perhaps the *awd* polypeptide plays a role in the protein of which it is a subunit, that is analogous to the role of subunit V in yeast cytochrome oxidase.

Materials and methods

Drosophila strains

The *ca K-pn* and *pn*; *kar e* stocks were obtained from the Bowling Green Stock Center. The *mwh red e*, *Canton S* and *y*;

GI DTS(3)2/y⁺, TM3 Sb Ser stocks are maintained in this laboratory. The *awd^{bs}* allele (Dearolf et al. 1988a) was generated in a *mwh red e* chromosome, by a hybrid dysgenic cross and maintained as *mwh red e awd^{bs}/TM3, Sb Ser* or as *y; mwh red e awd^{bs}/y⁺, TM3 Sb Ser*. To generate *K-pn* revertants, *ca K-pn* males were irradiated with gamma rays from the cesium source of a Gammator B (Isomedix Inc., Parsippany, New Jersey) with a total dose of 4000 R. The irradiated males were then mated to *pn;kar e* females. The *pn* male progeny of this cross represent putative *K-pn* revertants. The revertants were maintained as *y; ca awd^{KR}/y⁺, TM3 Sb Ser*. For a description of markers and balancers used in this report see Lindsley and Grell (1968).

Stage of lethality

The stage of lethality was determined for revertant-bearing chromosomes as homozygotes and as transheterozygotes with each other and with *mwh red e awd^{bs}*. Eggs were collected from balanced lethal stocks or from crosses between different stocks. All of the larvae from a vial were harvested and the number of homozygous (or transheterozygous) and heterozygous larvae was counted. The homozygous or transheterozygous larvae could be distinguished from heterozygous larvae because they expressed the *y* larval phenotype. The homozygous or transheterozygous larvae were placed in fresh vials and their further development was observed. Some were dissected to examine their imaginal disc phenotype. Lethal *pn; awd^{K-pn}/awd⁺* male larvae were produced by crossing homozygous *pn* females to homozygous *awd^{K-pn}* males. Male larvae were recognized by the presence of testes.

Genomic Southern blots

Flies were homogenized in 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 1% sarcosyl, then NaCl and Proteinase K were added to final concentrations of 0.1 M and 0.1 mg/ml, respectively. The homogenate was incubated at 37°C for 1 hr, then extracted once with 1 : 1 phenol/chloroform. The DNA in the aqueous phase was precipitated with ethanol, and the pellet resuspended in 0.1 × SSC. RNase was added (to 10 µg/ml), and the sample incubated 30 min at 37°C; proteinase K was added (final concentration of 0.1 mg/ml) and the incubation continued for 1 hr. The sample was extracted with phenol/chloroform and precipitated, and the pellet was resuspended in TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]. The DNA was digested with restriction enzymes, fractionated on 0.8–1.5% agarose gels, blotted onto nylon membranes with 20× SSC, fixed by UV-irradiation, and hybridized to radioactive DNA probes. A solution of 5× SSC, 5× Denhardt's solution, 0.1% SDS, and 250 µg/ml denatured calf thymus DNA was used for prehybridization and hybridization at 65°C. Filters were washed for 30 min at 65°C in 2× SSC with one change of solution, for 30 min at 65°C in 2× SSC, 0.1% SDS, and for 10 min at 65°C in 0.1 × SSC. The filters were blotted dry and autoradiographed.

Northern analysis

Total RNA was isolated by a modification of the method of Thireos et al. (1980). Adults or larvae were homogenized in a 1 : 1 mixture of phenol and RNA extraction buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.5% SDS]; the aqueous phase was extracted several more times with a 1 : 1 mixture of phenol/chloroform. RNA was precipitated from the aqueous phase with 2.5 volumes of ethanol overnight at 20°C and pelleted by spinning for 40 min at 16,000g. The RNA was washed with 70% ethanol and resuspended in water. For

Northern analysis, RNA samples were prepared in running buffer plus 2.2 M formaldehyde and 50% (vol/vol) formamide, and heat-denatured 5 min at 60°C. The RNA was fractionated on agarose gels containing 2.2 M formaldehyde [running buffer: 20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA]. The RNA was blotted onto a nylon membrane with 20× SSC and hybridized to radiolabeled probes. The prehybridization and hybridization solution was 5× SSC, 50% (vol/vol) formamide, 5× Denhardt's solution, 0.1% SDS, and 250 µg/ml denatured calf thymus DNA. The filters were washed three times in 2× SSC, 0.1% SDS at room temperature, and twice in 0.1× SSC and 0.1% SDS at 50°C.

Germ-line transformation

Embryos were injected according to the method of Rubin and Spradling (1982) with a plasmid containing the *awd* gene cloned from a *K-pn* genomic library. The embryos injected were the progeny of a cross between homozygous *ry* females and males of the genotype *ry e awd^{bs}/TM3, ry e Sb* (Fig. 6). Injected embryos that gave rise to flies with a *ry⁺* phenotype (*G₀* individuals) were mated to flies with the genotype *ry e awd^{bs}/TM3, ry e Sb*. To establish transformed lines, *G₁* individuals with a *ry⁺* phenotype were mated to flies with the genotype *ry e awd^{bs}/TM3, ry e Sb*. By examining the progeny of this cross and comparing the segregation of the *ry⁺* phenotype to that of the *e* and *Sb* phenotypes, it was possible to determine that transformed lines carried the *awd^{K-pn}* insert on the third chromosome. To test whether such inserts confer the *K-pn* phenotype, females homozygous for *prune* were mated to transformed males with the genotype *ry⁵⁰⁶ [ry⁺ awd^{K-pn}]/TM3, ry⁵⁰⁶ e Sb*. If the insert confers the *K-pn* phenotype, then no male progeny with the genotype *pn; ry⁵⁰⁶ [ry⁺ awd^{K-pn}]/+* should survive.

Gel filtration chromatography

Approximately 5.0 grams of *Drosophila* embryos were homogenized in 5.0 ml of buffer as used by Arrigo (1987) for subcellular fractionation [10 mM Tris (pH 7.4), 10 mM NaCl, 5 mM MgCl₂, 0.2% Triton X-100, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride]. Homogenization was performed at 0–4°C with a Wheaton glass homogenizer (B pestle). The homogenate was centrifuged at 12,000g for 15 min to pellet unbroken cells, nuclei, and large organelles. This supernatant was lyophilized to one-tenth starting volume and loaded onto a Bio-Gel P300 column. Proteins were eluted from the column with 10 mM Tris (pH 7.4). Fractions were collected (1 ml), lyophilized to dryness, resuspended in 150 µl of SDS sample buffer, boiled for 5 min, and electrophoresed on 12% SDS–polyacrylamide gels. The gels were either stained with Coomassie brilliant blue R or used for Western blots with antiserum from a rabbit immunized against an *awd*–*trp E* fusion protein.

Immunological methods

An *AluI* fragment of an *awd* cDNA (pc600) containing codons 48–139 was inserted into the *SmaI* site of the *PATH-2* expression vector (T.J. Koerner, unpubl.). The fusion protein produced by this construct contains 13 kD of *awd* protein fused to the carboxyl terminus of the *Escherichia coli trp E* protein. This fusion protein was isolated from a preparative SDS–polyacrylamide gel, ground to a powder in liquid nitrogen and resuspended in 50% (vol/vol) Freund's adjuvant. Once a week for 3 weeks 0.2 ml of this mixture was injected into a New Zealand white rabbit. A final boost injection was given 2 weeks later. One week after the last injection, about 30 ml of blood was collected

and allowed to clot overnight at 4°C. After removing the clot, the remaining liquid was centrifuged at 10,000 rpm for 10 min to remove any remaining cells from the serum. This serum was used for Western blots.

Whole larvae (40) were homogenized in 0.5 ml of SDS sample buffer [20% glycerol, 3% SDS, 3% β -mercaptoethanol, 5 mM Tris-HCl (pH 7.4)]. The homogenate was boiled for 5–10 min and 40 μ l was loaded into each lane of an SDS–polyacrylamide gel (3.5% stacking gel, 12% separating gel). After fractionation, the larval proteins on the gel were transferred to a sheet of nitrocellulose by electroblotting for 2 hr at 200 mA in transfer buffer (20% methanol, 25 mM Tris base, 192 mM glycine). The nitrocellulose was blocked with a solution of 5% powdered nonfat milk, 0.9% NaCl, 10 mM Tris-HCl (pH 7.4), and 0.04% azide for 2 hr at 4°C. Reactions with antiserum and [125 I]protein A were performed in a solution of 2% powdered nonfat milk, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.25% gelatin, 0.05% NP-40, and 0.04% azide. After the reactions the filter was washed for 1 hr in four changes of 1.0 M NaCl, 0.4% sarcosyl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.25% gelatin, and 0.04% azide. The filter was rinsed with water, blotted dry, and autoradiographed.

DNA sequencing

The cDNA clone *pc600* was sequenced in both directions by the method of Maxam and Gilbert (1980). The mutant genomic DNA subclone *pEco6.2* from the *Hind*III site of the inserted P element to beyond the presumed 5' end of the *awd* transcription unit was also sequenced. Dideoxy sequencing (Sanger et al. 1977) of *pc600* resolved ambiguities near each of the termini of the cDNA clone.

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Note

Sequence data presented in Figure 7 have been submitted to the EMBL/GenBank Data Libraries under accession number Y00226.

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