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 awdK14 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; awdK-pn/awdK 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.

[Key Words: Cell lethal mutation; gene expression; gene interactions]

Received May 9, 1988; revised version accepted August 18, 1988.

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, awdB3. The wing discs of third-instar mutant larvae are small and have extensive areas of cell death; the brains and ventral ganglia of awdB3 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 awdB3 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 awdB3 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 awdB3. This suggested that K-pn and awdB3 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
Mapping the K-pn revertants awdKR7, awdKR14, and awdKR17

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 diagramed in Figure 4. The awd gene in both control [mwh red e] and K-pn flies is contained in a 2.2-kb PstI fragment, while the corresponding PstI 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 PstI fragment from the K-pn chromosome are detectable in the DNA from heterozygous revertant flies with the genotype awdKR7/TM3. As shown in Figure 3A (lane 8) and more clearly in Figure 3B (lane 3), the 2.2-kb PstI fragment from the K-pn chromosome is missing in awd KRT. Otherwise, the DNA fragments generated by restriction enzyme digestion of awdKRT/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 awdKRT.

In awdKRT7, the 2.2-kb PstI 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 SalI fragment contains the 2.2-kb PstI 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 SacI site close to the 3' end of the awd gene was removed by the awdKRT7 deletion. The results localize the 0.8-kb deletion in awdKRT7 toward the 3' end of the awd gene, as shown in Figure 4.

The initial whole genome Southern blots of awdKRT14/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 1. Screen for K/Her 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.

Figure 2. Salivary gland chromosomes from awdKRT21/+ larvae. The deletion of the 100C-D region is indicated by the arrow.
membrane. The membranes were hybridized to a radiolabeled fragment from adults which were digested with AluI, which cuts at the tetranucleotide sequence AGCT.

Figure 3. Analysis of awd* revertant alleles awdKR14, awdKR16, and awdKR17 by whole genome Southern blots. DNA from adults was digested with SalI (A, lanes 1–5), PstI (A, lanes 5–10; B, lanes 1–5), or AluI (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, 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 awdKR14/awdKR17 is one-half that of the others because awdKR17 deletes the region which codes for that transcript. We used awdKR14/awdKR17 transheterozygotes in this experiment because a second mutation elsewhere on the awdKR17 chromosome makes it impossible to get homozygous awdKR17 third-instar larvae. The 0.8-kb awd transcript is present in Canton-S RNA, but is missing in both awdKR14/awdKR16 and awdKR14/awdKR17 RNA (Fig. 5A). The 0.9-kb transcript is absent in awdKR14/awdKR17, but is present at normal levels in awdKR14/awdKR16 (Fig. 5B). The only transcript missing from awdKR14/awdKR16 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 SalI 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 SalI fragment rescues the lethality caused by awd3 [data not shown]. We used a ry+ transformant with an insertion on the third chromosome to demonstrate that this 3.4-kb SalI fragment is sufficient to cause the lethal interaction with prune. The genotype of this transformant is ry506 [ry+ awdKR14]/TM3, ry506 eSb.

awdKR14 AluI 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, awdKR14 and awdKR17, on the accumulation of the awd gene transcript

To assess the effect of awdKR14 and awdKR17 on the transcription of awd and nearby genes, RNA was isolated from Canton-S, awdKR14/awdKR14, and awdKR17/awdKR17 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 awdKR14/awdKR17 is one-half that of the others because awdKR17 deletes the region which codes for that transcript. We used awdKR14/awdKR17 transheterozygotes in this experiment because a second mutation elsewhere on the awdKR17 chromosome makes it impossible to get homozygous awdKR17 third-instar larvae. The 0.8-kb awd transcript is present in Canton-S RNA, but is missing in both awdKR14/awdKR16 and awdKR14/awdKR17 RNA (Fig. 5A). The 0.9-kb transcript is absent in awdKR14/awdKR17, but is present at normal levels in awdKR14/awdKR16 (Fig. 5B). The only transcript missing from awdKR14/awdKR16 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.

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lethality of awd3; some change in the awd gene must have occurred. To find this change, genomic DNA from mwh red e, K-pn, and awdKR14 larvae was digested with AluI, 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 awdKR14 revertant DNA when probed with the awd cDNA, but when probed with the upstream sequence, two new bands appear in the
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+K1K1, awd K1K1, and awd K1K2. 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.

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 ry506[ry+ awdK-pn] chromosome were recovered (Fig. 6). This indicates that even in the presence of two copies of the awd+ gene, one copy of awdK-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 awdK1K1 and awdK1K2 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. 51 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 bs 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][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 51 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 is 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 the 1.35-kb transcript and the 0.8-kb transcript. The size of this intron was estimated from the difference in size between the 1.35-kb transcript and the 0.8-kb transcript.
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. The most favorable context for initiation of translation is the sequence ACCATGG. In awd, this context 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 internal 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 awdB3 homozygous larvae (Fig. 8).

Although no awd transcript is detectable in awdRK14 larvae (Fig. 5), awdRK14 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, awdB3, causes lethality at the early pupal stage. Since the protein coding region is not affected by awdRK14, we considered the possibility that homozygous awdRK14 larvae survived to a later stage than do homozygous awdB3 larvae because they accumulate a small amount of awd protein. To examine this possibility, we compared the level of awd protein in homozygous awdRK14 larvae to that of control larvae. In homozygous awdRK14 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

![Figure 6. Germ-line transformation of Drosophila embryos with awdRK14 DNA. The embryos from a cross (P) between ry/ry and ry e awdB3/ TM3, ry Sb e flies were injected with a DNA fragment containing the K-pn allele of awd. A 3.4-kb Sall 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 awdB3/ry e awdB3 genotype, proving that the K-pn allele of awd can rescue the awdB3 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.](https://genesdev.cshlp.org/content/2/6/1337/F6)

![Figure 7. Diagram of the awd gene.](https://genesdev.cshlp.org/content/2/6/1337/F7)
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 b3 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 awd<sup>B3</sup> larvae. Third-instar larvae (approximately 1 larva/10 µl buffer) were homogenized in SDS sample buffer, the homogenate was boiled, and 40 µl 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 [<sup>125</sup>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<sup>KR-14</sup>] larvae homozygous for awd<sup>KR-14</sup>. (B) [awd<sup>K<sub>pe</sub></sup>] Larvae homozygous for awd<sup>K<sub>pe</sub></sup>, [pn] larvae homozygous for pn; [pn;awd<sup>K<sub>pe</sub></sup>] male larvae hemizygous for pn and heterozygous for awd<sup>K<sub>pe</sub></sup>. (C) ash<sub>1</sub> III-10 is a late larval lethal included as a control for the effects of larval death.
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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 [125I]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.

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 Neurospora crassa cytochrome oxidase subunit V is taken from Power et al. [1984]. Amino acids that are identical or similar are shaded.

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 recovered failed to complement the lethality of awdR3. We examined the nature of three cytologically normal revertants at the molecular level. One of the three, awdKR7, is a deletion that removes sequences flanking the awd gene in addition to the entire awd gene (Fig. 4). Another, awdKR17, is missing 800 bp which include the 3' transcribed region of the awd gene and sequences flanking a downstream gene (Fig. 4). The awdKR17 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 awdKR14 because it only affects the awd transcript. No awd transcript was detected in third-instar awdKR14 homozygous larvae (Fig. 5). The awdKR14 mutation occurred in the 5' nontranscribed region of the awd gene (Figs. 3 and 4).
Thus, awdKR14 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 awdK-pn.

The phenotype of awdKR14 homozygotes and awdbs3/awdKR14 transheterozygotes is less severe than that of awdbs3 homozygotes. This less severe phenotype suggested that awdKR14 is not a null allele. Southern and Northern blot analyses showed that awdKR14 affects an upstream regulatory element of the awd gene. Although we were not able to detect accumulation of the awd transcript in awdKR14 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 awdKR14 to be a consequence of its reduced accumulation of the abnormal awdK-pn gene product. The amount of awdK-pn polypeptide in awdKR14/awdKR14 homozygous larvae is adequate to support development until the pharate adult stage, but the amount made in awdKR14/awd+ heterozygous larvae is too low to cause the lethal interaction with pn.

Interaction of pn with awdK-pn

The awdK-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; awdK-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 awdK-pn reduces the accumulation of both the awdK-pn polypeptide and the awd+ polypeptide. It appears that a certain amount of the awdK-pn abnormal polypeptide is required for the lethal interaction with pn to occur. Male larvae with the genotype pn; awdK-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; awdK-pn/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; awdKR14/awd+ would be expected to have a 1 : 5 ratio of mutant to wild-type polypeptide, because the awdKR14 allele produces only 20% as much of the awdK-pn polypeptide as does the awdK-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 awdK-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 awdK-pn.

The relationship between the pn product and the pn; awdK-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 Pn 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 awdK-pn is quite specific; awdK-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 awdK-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]/y+, TM3 Sb Ser stocks are maintained in this laboratory. The awdR allele [Dearolf et al. 1988a] was generated in a mwh red e chromosome, by a hybrid dysgenic cross and maintained as mwh red e awdR/TM3, Sb Ser or as y; mwh red e awdR/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 awdR/+ 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 homogametes and as transheterozygotes with each other and with mwh red e awdR. 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; awdR+/awd + male larvae were produced by crossing homozygous pn females to homozygous awdR+ 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 extracted several more times with a 1 : 1 mixture of phenol and RNA extraction buffer [10 mM Tris-acetate (pH 7.5), 5 mM sodium acetate, 1 mM EDTA, 0.2% Triton X-100, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride]. 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 a 12% SDS–polyacrylamide gel. The gels were either stained with Coomassie brilliant blue R or used for Western blots with antiserum from a rabbit immunized against an awdR fusion protein. 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 awdR/TM3, ry e Sb (Fig. 6). Injected embryos that gave rise to flies with a ry+ phenotype (Cio individuals) were mated to flies with the genotype ry e awdR/TM3, ry e Sb. To establish transformed lines, G1 individuals with a ry+ phenotype were mated to flies with the genotype ry e awdR/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 awdR+ insert on the third chromosome. To test whether such inserts confer the K-pn phenotype, females homozygous for prone were mated to transformed males with the genotype ry5000 [ry+ awdR+]/TM3, ry5000 + Sb. If the insert confers the K-pn phenotype, then no male progeny with the genotype pn; ry5000 [ry+ awdR+]/+ 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 (β 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 a 12% SDS–polyacrylamide gel. 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 Smal 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 μl 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 for isolating some cytochrome C oxidase subunits. Dideoxy sequencing (Sanger et al. 1977) of pc600 resolved ambiguities near each of the termini of the cDNA clone.

Acknowledgments

This work was supported by a grant to A.S. from the National Institutes of Health. J.B. was supported by a postdoctoral fellowship from the National Institutes of Health. We would like to thank T.J. Koerner for the use of the PATH plasmids which he constructed, our colleagues in Douglas Fambrough’s lab for instruction in immunological methods, and Karen Drummey for isolating some K-pn revertants.

Note

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

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*Genes Dev.* 1988, 2:
Access the most recent version at doi:10.1101/gad.2.10.1333