Germline Transformation Using a *prune* cDNA Rescues *prune/Killer of prune* Lethality and the *prune* Eye Color Phenotype in *Drosophila*

Lisa Timmons and Allen Shearn

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

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ABSTRACT

Null mutations in the *prune* gene of *Drosophila melanogaster* result in prune eye color due to reductions in red pigment accumulation. When one copy of the $awd^{Killer\ of\ prune}$ mutant gene is present in a *prune* background, the animals die. The cause of *prune/Killer of prune* lethality remains unknown. The genomic region characterized for the *prune* locus is transcriptionally active and complex, with multiple and overlapping transcripts. Despite the transcriptional complexity of the genomic region of *prune*, accumulated evidence suggests that the *prune* locus is small and consists of a single transcription unit, since every *prune* allele to date exhibits both prune eye color and *prune/Killer of prune* lethality. A functional *prune* product from a single, full-length cDNA was identified in this study that can rescue both the eye phenotype and *prune/Killer of prune* lethality. The DNA sequences of several mutant *prune* alleles along with Western blot analysis of mutant proteins provide convincing evidence that *prune* mutations are nulls, and that the cDNA identified in this study encodes the only product of the *prune* locus.

THE brick red eye color of wild-type *Drosophila melanogaster* is a composite of two classes of pigments, the ommochromes and the pterins, which are deposited in membrane-bound pigment granules in primary and secondary pigment cells of each ommatidium (SHOUP 1966). Ommochromes, originally isolated from insect ommatidia, are brown pigments containing the structural group 1,2-pyridino-3hydroxy-phenoxazine and are biosynthesized from tryptophan. Eyes containing only ommochromes appear dark brown. Pterins, originally isolated from butterfly wings, are derivatives of 2-amino-4(3-hydroxy)-oxopteridine and are biosynthesized from purines, specifically GTP. Eyes containing only pterins appear bright red (PHILLIPS and FORREST 1980). Fluorescent and pigmented compounds in the Drosophila eye are separable by TLC and include the following: the orange-yellow ommochrome 3-hydroxy-kynurenine, the yellow pteridines sepiapterin and deoxysepiapterin, fluorescent purple isoxanthopterin, fluorescent blue pterin, biopterin, and xanthuronic acid, and the bright red pigments collectively called drosopterins (FERRE *et al.* 1986).

The *prune* mutants of Drosophila are recessive, sex-linked, nonlethal eye color mutants with brownish purple eyes (LINDSLEY and ZIMM 1992). The levels of ommochrome pigments measured in *prune* mutant eyes are normal (110% of wild type), while the levels of drosopterin pigments measured in *prune* mutant eyes and whole adult bodies of *prune* mutants are reduced to 25% of wild type (SCHWINCK 1975; EVANS and HOWELLS 1978; FERRE *et al.* 1986). This reduction in *prune* eyes is observed for four members of the red pigment class that have been identified to date (drosopterin, isodrosopterin, neodrosopterin, and aurodrosopterin), but not all members are reduced to the same level. Neodrosopterin is the most abundant red pigment in *prune* eyes, while aurodrosopterin is reduced to the lowest level (FERRE *et al.* 1986). *prune* mutants are also reported to have higher than normal uric acid content at all stages (LIFSHYTZ and FALK 1969; HACKSTEIN 1975).

The metabolic aberrations noted above do not adversely affect *prune* flies: homozygous mutants are viable and fertile, and appear wild type except for the eye color. However, in the presence of one (or more) copies of *Killer-of-prune* (awd^{Kpn}), homozygous or hemizygous *prune* mutations are lethal. This lethal *prune/Killer of prune* genetic combination was first noted when no male progeny were obtained from a cross of females homozygous for *prune* mutations on the *X* chromosome and males homozygous for awd^{Kpn} (STURTEVANT 1956; OREVI and FALK 1975). The awd^{Kpn} mutation in a $prune^+$ background is homozygous viable and fertile and has no mutant phenotype except for reduced fecundity at elevated temperatures (27–30°).

awd^{Kpn} is a mutant allele of the *abnormal wing discs* (*awd*) gene that is located on the third chromosome (DEAROLF *et al.* 1988a). The *awd* gene encodes a nucleoside diphosphate kinase, NDP kinase, (BIGGS *et al.* 1990) that catalyzes the reversible conversion of nucleoside diphosphates to nucleoside triphosphates (PARKS and AGARWAL 1973). Homozygosis for null *awd* alleles causes lethality at the end of the third larval instar. The awd^{Kpn} allele has a single amino acid substitution of

Corresponding author: Allen Shearn, Department of Biology, The Johns Hopkins University, Baltimore, MD 21218.
E-mail: bio_cals@jhu.edu

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proline to serine at amino acid 97 in the 17-kD subunit of the AWD protein hexamer (Lascu *et al.* 1992; Timmons *et al.* 1995) This substitution does not dramatically affect the enzymatic activity of the KPN protein: *awd*^{Kpn} homozygotes have about one-third the NDP kinase specific activity of wild-type individuals, which is still four times more than the specific activity required for viability (Timmons *et al.* 1995). *awd*^{Kpn} individuals are viable and fertile and appear wild type.

Lethal *prune/Killer of prune* individuals live for quite a prolonged period of time in the third larval instar stage (over 3 weeks) and eventually die. The animals acquire melanotic tumors during this stage and the animals become transparent as fat body utilization and histolysis continues. Even though *prune/Killer of prune* individuals do not pupate, their imaginal discs are capable of differentiating when transplanted into wild-type, metamorphosing hosts (E. Hersperger, unpublished results). In contrast, imaginal discs from individuals homozygous for the null allele of *awd*, *awd*^{KRs6}, or for the severe hypomorphic allele of *awd*, *awd*^{b3}, do not differentiate when transplanted (Dearolf *et al.* 1988a; E. Hersperger, unpublished results). This and other observations (Timmons *et al.* 1995) refute the notion that an AWD enzyme deficiency contributes to lethality of *prune/Killer of prune* individuals. Why this combination of otherwise viable mutations is lethal and how only one copy of the *awd*^{Kpn} gene causes lethality of *prune* flies has remained a mystery since the discovery of the lethal interaction in 1956.

One important, yet still lacking, key to the understanding of the *prune/Killer of prune* interaction is the function of the *prune* gene. What complicates this matter further is that two groups have reported two different sequences for the *prune* gene: Teng *et al.* (1991) identified a transcript encoded in one exon while Frolov *et al.* (1994) identified a transcript encoded in two exons. The second exon of Frolov *et al.* (1994) corresponds to the Teng *et al.* (1991) exon. Even in the region of overlap between the two reported transcripts there are disagreements in the reported nucleotide sequences that result in reading frame discrepancies between the conceptual protein sequences. Both groups reported a DNA rearrangement at the putative *prune* locus in *prune*^{38}, a *P*-element insertion allele, and Teng *et al.* (1991) showed that in a *prune*^{38} revertant, the genomic DNA had a wild-type restriction pattern. This is an indication that both groups have indeed identified at least a part of the *prune* locus.

To understand the function of the Prune protein, it is imperative to identify the full length *prune* transcript and to know the correct primary amino acid sequence of the protein. We have determined the nucleotide sequences of a *prune* cDNA and *prune* genomic DNA. We have verified that the cDNA encodes a functional Prune product by demonstrating that it can rescue both the *prune* eye phenotype and the *prune/Killer of prune* interaction.

MATERIALS AND METHODS

Stocks: Flies were reared on a yeasted cornmeal-agar-molasses medium at 21–24°. Heat shocks were performed by inserting media vials containing larvae or adults into a 37° water bath for 1 hr. *y w*^{67C}, *Canton S*, *ca awd*^{Kpn}, and *prune; stw kar e* stocks were obtained from the Bloomington Stock center. *y prune*^{38} was recovered in a dysgenic screen by Robertson *et al.* (1988) and provided to us by Hugh Robertson. *y f prune*^{77C33} was obtained from Mel Green. *y prune*^{A}, *y prune*^{12C}, and *y prune*^{18a} were generated in this lab by EMS mutagenesis of *y* males. *prune*^{1}, *prune*^{2}, *prune*^{3}, and *prune*^{pw2} were provided to us by Dr. Tadmiri Venkatesh.

Transformation/heat-shock rescue plasmids: The *prune* cDNA insert for constructing the transformation plasmid pHS-PN^{+}3 was prepared by amplification from an adult random-primed cDNA library in Lambda ZapII (Stratagene) using oligos #043 (CCCGGGCATATGTGCTTTCTACGATTTTTGGCC) and # 037 (GCCTGGATCCTTATTAAGAGAGTCCCAGCTGCGGCT). This insert was shuttled into pCRII cloning vector (Invitrogen) to create pPN1 and then cloned as an *Eco*RI-*Bam*HI fragment into pCaSpeR-HS-act (Thummel *et al.* 1988) to produce pHS-PN^{+}3. The insert for constructing plasmid pHS-PN^{+}4 was similarly made by amplification from *prune* cDNA-containing plasmid pTcD37 (Teng *et al.* 1991) using oligos #038 (CCCGGGCATATGGGCAACG AATCGTGTGACTTG) and #037, shuttling into pCRII to create pPN2, and cloning as an *Eco*RI-*Bam*HI fragment into pCasPeR-HS-act. pHS-PN^{+}3 and pHS-PN^{+}4 were purified on Qiagen maxi columns and injected into manually dechorionated *y w*^{67C}; *ca awd*^{Kpn} embryos with transposase source pπ25.7wc (Karess and Rubin 1984) using standard procedures (Spradling and Rubin 1982). Four *y w*^{67C}; *ca awd*^{Kpn} *(pHS-pn*$^{+}$*3)*-transformed stocks were obtained (A–D), each with single inserts on the second chromosome. Seven *y w*^{67C}; *ca awd*^{Kpn} *(pHS-pn*$^{+}$*4)*-transformed stocks were obtained: lines A, C, E, and G have single insertions on the *X* chromosome (line C is also homozygous lethal); lines B, D, and F have single insertions on the second chromosome. Those transformed lines with insertions on the second chromosome were also maintained in a *y w*^{67C} background by crossing out the *awd*^{Kpn} chromosome.

Genomic prune sequencing plasmid: A genomic fragment containing the *prune* gene was amplified from Canton S DNA using oligos #043 and #037 and cloned into pCRII (Invitrogen) to produce plasmid pPN9.

Sequencing: Dideoxy chain termination sequencing was performed on double-stranded plasmid DNA with USB Sequenase version 2 using 42° reaction temperature or with the Amplitaq cycle sequencing kit (Perkin Elmer) using a 55° annealing temperature and 72° elongation temperature. Sequencing of *prune* alleles was performed on PCR-generated products. *prune*^{2} was sequenced from three independent isolates, *prune*^{12C} and *prune*^{18a} from two independent isolates and *prune*^{3}, *prune*^{77C33}, *prune*^{38}, *prune*^{A} and *prune*^{pw2} from one isolate.

Overexpression and purification of Prune protein: The Prune coding region from pPN1 was inserted as a *Nde*I-*Bam*HI fragment into pVex II expression vector (obtained from Sankar Adhya, Lab of Molecular Biology, National Cancer Institute, National Institutes of Health) to produce plasmid pPN^{+}7. Calcium-competent *BL21* cells were transformed with pPN^{+}7. At an OD_{595} = 0.4, Prune protein expression was induced with isopropylthiogalactose at a final concentration of 0.5 mM for 4 hr. The overexpressed Prune protein was localized to inclusion bodies under these and a variety of other induction conditions tested. Overexpressed Prune was soluble in 4 M guanidine HCl, 6 M urea, or in 0.5% SDS, and precipitated when diluted. Six hundred milliliters of overexpressed cell culture was used to purify Prune protein. The cell pellet was resuspended in HBB [25 mM HEPES pH 7.9, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT)] at

1/100 original culture volume and lysed by lysozyme treatment and sonication. Guanidine HCl was added to the lysed cell suspension to a final concentration of 6 M. The suspension was sonicated, incubated at 4° for 1 hr, and then centrifuged for 30 min at 10,000 × *g* to remove debris. The supernatant was then diluted to a final guanidine concentration of 4 M, sonicated, incubated at 4° for 1 hr, and centrifuged. The supernatant was diluted to 1.5 M guanidine, sonicated, incubated at 4° for 1 hr, and centrifuged. The washed Prune pellet was resuspended in HBB/6 M guanidine and the series of guanidine washes was repeated. The guanidine-washed pellet was then solubilized in 5% SDS, incubated at room temperature for 30 min, and centrifuged. The resulting supernatant was diluted 1:10, incubated, and centrifuged. The final insoluble pellet was washed extensively with distilled water. The Prune protein was solubilized in 6 M urea then eluted from a Sephadex G-150 column (Pharmacia) and concentrated by precipitation in distilled water and subsequent centrifugation. Prune was then eluted from a Sephadex G-150 column in 0.7% SDS/1.5 M β-mercaptoethanol/25 mM HEPES pH 7.9 and precipitated by dilution in distilled water and centrifugation. The Prune protein was then washed extensively in distilled water and resuspended in RotoLytes (BioRad)/6 M deionized urea and resolved by preparative isoelectric focusing in a BioRad Rotofor preparative IEF cell. The Prune fractions were pooled and concentrated by dilution/centrifugation. Further purification was achieved by running the processed Prune protein on preparative SDS 10% polyacrylamide gels, staining with Coomassie blue, eluting Prune from gel slices in elution buffer (2% SDS, 1 mM DTT, 2 mM EDTA, 100 mM NaCl, 20 mM Tris pH 6.8) overnight, and concentrating by acetone precipitation. Purified protein was resuspended in elution buffer and injected into rabbits.

Preparation and purification of anti-Prune antibody: A Prune affinity column was prepared by resuspending Prune protein in 1% SDS/coupling Buffer (100 mM $NaHCO_2$, 500 mM NaCl pH 8.8) and coupling to CNBr-Sepharose 4B (Sigma) as described (TIMMONS *et al.* 1995). Rabbit antisera was diluted 1:2 in TBS (20 mM Tris pH 7.5, 0.5 M NaCl) and loaded onto the column. The column was then washed in TBS and anti-Prune antibodies were eluted in 0.5% acetic acid (0.1 M)/0.15 M NaCl and immediately neutralized. Prune adsorption strips were prepared by separating purified Prune protein (described above) on SDS 10% polyacrylamide gels, electroblotting the separated proteins onto PVDF membranes (Millipore), and trimming the Ponceau S-stained membrane of all but the Prune band. The column-purified antibodies were incubated with the Prune adsorption strips overnight. The strips were rinsed with PBS, and antibodies were eluted from the strips in 0.2 M glycine/1 mM EGTA pH 2.8 (SAMBROOK *et al.* 1989) and immediately neutralized with 1/10 volume 1 M Tris and 1/10 volume 10× PBS pH 7.5. The purified anti-Prune antibody eluates were pooled, concentrated, and diluted 1:1000 before use.

Western blot analysis: Protein concentrations were determined by Bio-Rad Protein Assay. Samples were boiled extensively in sample buffer containing β-mercaptoethanol and loaded onto SDS 10% polyacrylamide gels in either a Hoeffer or Idea Scientific protein gel apparatus. Proteins were transferred onto PVDF membranes (Millipore) overnight at 50 mA (7–35 V) in standard Tris/Glycine buffer. The blots were blocked in 5% nonfat dry milk/PBS/0.05%Tween 20 (BLOTTO), then incubated with antibody diluted in BLOTTO overnight. The secondary antibody used for all experiments was donkey anti-rabbit IgG coupled to HRP (Amersham) and detection of secondary was by Chemiluminescence (Amersham).

RESULTS

Sequence analysis of putative *prune* cDNAs and corresponding genomic DNA: *prune* cDNAs have been isolated and sequenced independently in two different laboratories. The TENG *et al.* (1991) version describes a 1503-bp transcript encoded in only one exon with an open reading frame (ORF) predicting a 41-kD protein. The FROLOV *et al.* (1994) version describes a 1773-bp transcript encoded in two exons with an ORF predicting a 44.5-kD protein. The second exon reported by FROLOV *et al.* (1994) is identical to the single exon reported by TENG *et al.* (1991). The FROLOV *et al.* (1994) conceptual protein is larger by 40 amino acids at the aminoterminus. In addition the DNA sequences reported by these two groups have three discrepant regions with the result that 64 amino acids differ between the two reported conceptual proteins (Figure 1).

To determine if one or both of the sequences encodes a complete *prune* product, we first amplified the putative *prune* coding region from wild-type Canton S genomic DNA using primers designed according to the larger FROLOV *et al.* (1994) sequence (#043 and #037, Figure 1). A PCR product was obtained using these primers, and the size of the PCR product matched the description of the *prune* genomic region in FROLOV *et al.* (1994). The same primer set was used to amplify a *prune* cDNA from an adult cDNA library. A product of the size and sequence matching the description of FROLOV *et al.* (1994) was obtained. Plasmid pHS-PN$^+$3 was made from this cDNA fragment. Primers were also designed according to the putative coding region described by TENG *et al.* (1991) (#038 and #037, Figure 1) and used to amplify a product from an adult cDNA library and from plasmid pTcD37, which contains a putative *prune* cDNA (TENG *et al.* 1991). A PCR product of the expected size from both sources was obtained from this primer set, and plasmid pHS-PN$^+$4 was created from the pTcD37-derived PCR fragment.

Sequence analysis of the amplified *prune* regions confirms the finding that the *prune* gene contains two exons. The intron sequence and position deduced from a comparison of genomic and cDNA sequences are in agreement with that reported by FROLOV *et al.* (1994). An *Eco*RI site is positioned within the intron. The sequence of the intron preceding this *Eco*RI site was not resolved by FROLOV *et al.* (1994). Our sequence of the intron preceding this *Eco*RI site reads GTGA GAATTC; our sequence after the *Eco*RI site matches that of FROLOV *et al.* (1994).

The first region of DNA sequence discrepancy between the sequences of TENG *et al.* (1991) and FROLOV *et al.* (1994) occurs early in the second exon at position 58 (AGGCT *vs.* AGGCCT, Figure 1). Our reading of this sequence matches that reported by FROLOV *et al.* who correctly determined that this sequence is a site for restriction endonuclease *Stu*I. The insertion of an extra base does not cause a reading frame shift between the two reported sequences because in the TENG *et al.* (1991) version of the *prune* transcript, Region I is part of the 5′ untranslated region.

The second region of sequence discrepancy causes an inferred amino acid disagreement since the sequences

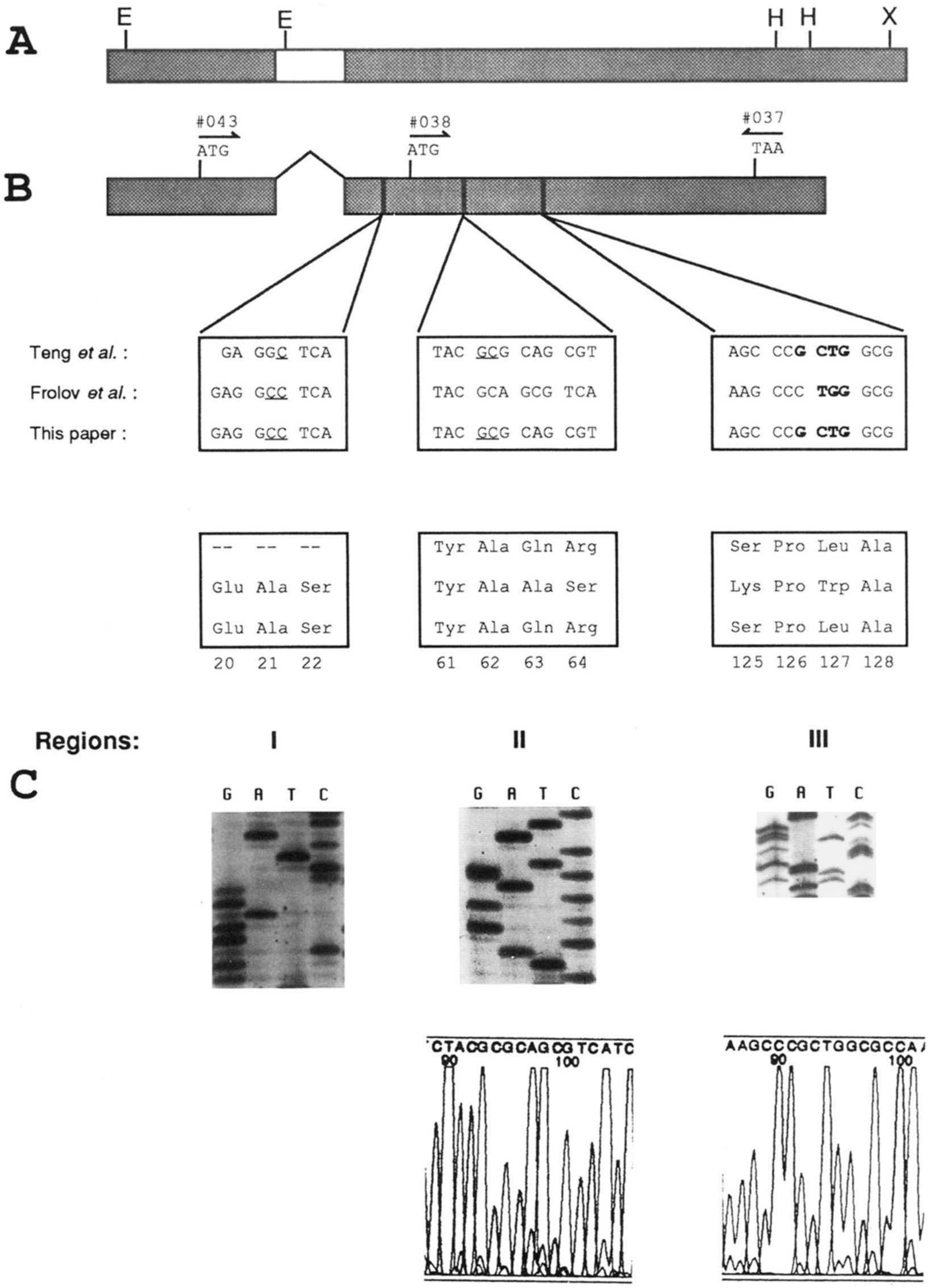


FIGURE 1.—Genomic arrangement at the *prune* locus. (A) Restriction map of ~2 kb of genomic DNA at the *prune* locus (TENG *et al.* 1991). E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. An *Eco*RI site is located within the intron, which is indicated by □. (B) Prune ORFs used in this paper. The longer reading frame was defined by FROLOV *et al.* (1994) and is contained within a cDNA (*c1.72*) represented by the entire boxed region. The shorter reading frame (second ATG) was defined by TENG *et al.* (1991) and is contained within a cDNA (*TcD37*) represented by the second stippled box. The longer ORF was amplified using oligos 043 and 037; the shorter ORF was amplified using oligos 038 and 037. Regions of sequence discrepancy are represented by filled rectangles and depicted in detail below them. (C) Sequencing gel data from this laboratory and automated sequencing data provided by D. TENG and T. VENKATESH for the discrepant regions.

differ by a frameshift in the codon for amino acid 63. Our inferred amino acid sequence at this region matches the amino acid sequence inferred by TENG *et al.* (1991). This region produced compressions on our sequencing gels and this is probably the source of the disagreement between the two reported sequences. To better resolve the compression, we sequenced at higher temperatures using Taq polymerase cycle sequencing. We present our sequence data from this method as well as automated sequencer readouts obtained from Dr. TADMIRI VENKATESH in Figure 1.

The third region of sequence discrepancy occurs in codon 124 in the second exon (C CCG CTG GCG *vs.* CCC TGG GCG). Our sequence of this region matches that reported in TENG *et al.* (1991), and our data is presented in Figure 1. The sequences and reading frames obtained by us, TENG *et al.* (1991), and FROLOV *et al.* (1994) are in agreement for the rest of the transcript region.

Rescue of *prune:Killer of prune* lethality: We wanted to determine if the *prune/Killer of prune* lethality could be rescued by expressing wild-type Prune protein in this otherwise lethal background. To define a *prune* transcript that would rescue the lethality, we placed intronless *prune* coding sequences (ATG to TAA) in the pCaSpeR-HS-actin transformation vector (THUMMEL 1988) that allows induction of mRNA through the hsp70 promoter and stability of induced RNA through the actin 3'UTR. pHS-PN$^+$3 is such a transformation plasmid containing the longer *prune* coding region defined by FROLOV *et al.* (1994); pHS-PN$^+$4 is a transformation vector with the shorter coding region defined by TENG *et al.* (1991) (Figures 1 and 2). The *prune P*-element transformants were maintained in a *y* w^{67C}; *ca* awd^{Kpn} background and were tested for the ability to rescue *prune/Killer of prune* lethality by performing Test Cross I (Figure 2B). Four transformation lines containing *pHS-PN*$^+$*3* insertions on the second chromosome were tested; three transformation lines containing *pHS-PN*$^+$*4* on the second chromosome were tested. The progeny of these test crosses were subjected to 37° heat pulses of 1 hr *per diem* for 1–5 days and the number and sex of the progeny reaching adulthood was tabulated. Each transformation line (four independent *pHS-PN*$^+$*3* lines, three independent *pHS-PN*$^+$*4* lines) was independently tested with the nine *prune* alleles listed in this paper. Each combination of *pHS-PN*$^+$*4* insert and *prune* allele gave similar results. Each combination of *pHS-PN*$^+$*3* insert and *prune* allele also gave similar results. The presence of male progeny from this cross is evidence of rescue of *prune/Killer of prune* lethality. A sample of the data is reported in Figure 2C.

No combination of heat shocks of *pHS-PN*$^+$*4* transformants provided rescue from *prune/Killer of prune* lethality. In contrast, all test crosses using *pHS-PN*$^+$*3* transformants produced viable male progeny, including test cross progeny receiving no heat shocks. Even with no heat shocks, the number of viable male progeny equaled the number of female progeny indicating complete rescue (Figure 2C). This is evidence that the HSP70 promoter is leaky under non-heat shock conditions, which has been noted by other investigators. We have also observed leakiness of the same promoter directing the *awd* cDNA in the pCaSper-HS vector (TIMMONS *et al.* 1995). Only a very small amount of Prune protein is produced from an uninduced HSP70 promoter from a single copy *pHS-PN*$^+$*3* insert (Figure 6, lanes 4 and 5). Nonetheless, this small amount of expression is adequate to rescue *Prune/Killer of prune* lethality.

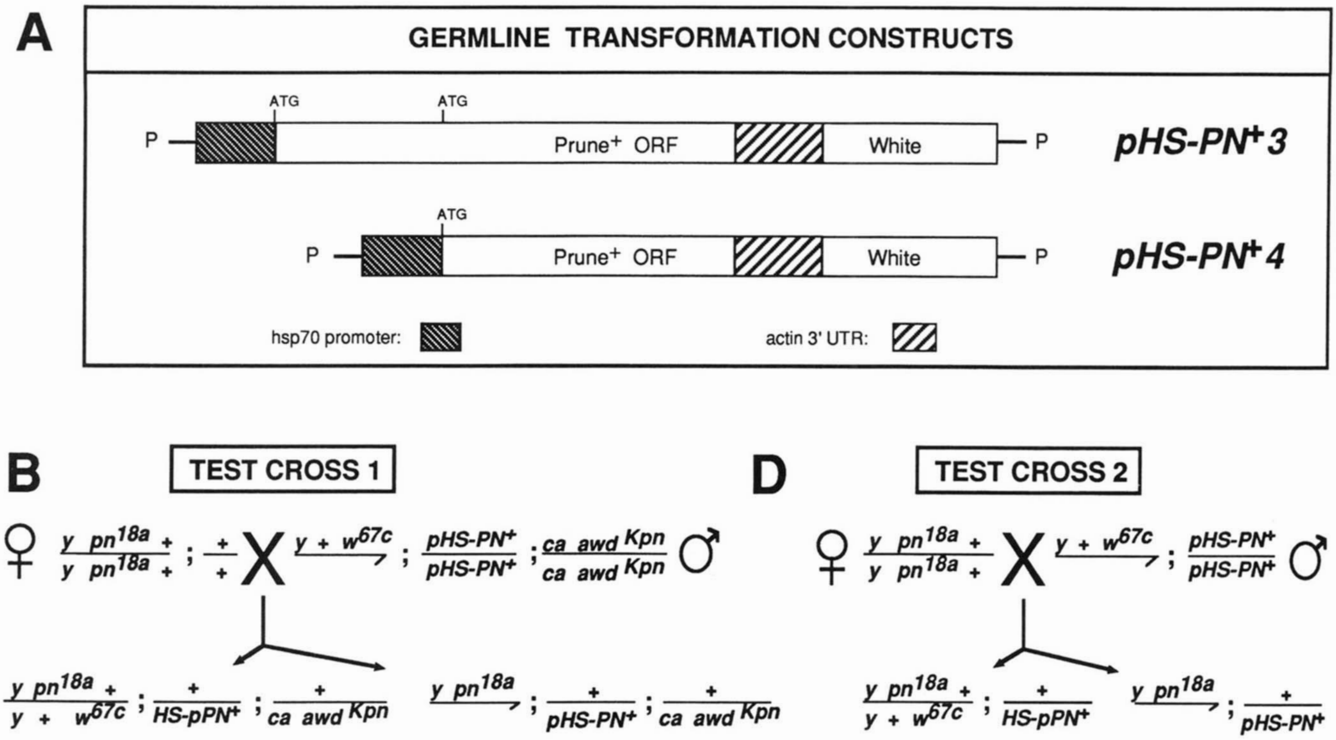
Rescue of prune eye phenotype: The ability of the two *prune* cDNAs to rescue the prune eye phenotype was assessed in Test Cross 2 (Figure 2D). In this test cross, males homozygous for *pHS-PN*$^+$*3* or *pHS-PN*$^+$*4* *P*-element inserts were crossed to females mutant for *prune,* but not *white.* Each transformation line (four independent *pHS-PN*$^+$*3* lines, three independent *pHS-PN*$^+$*4* lines) was tested with the nine *prune* alleles described in this paper. Each combination of *pHS-PN*$^+$*4* insert and *prune* allele gave similar results. Each combination of *pHS-PN*$^+$*3* insert and *prune* allele also gave similar results. Results from Test Cross 2 for only one combination of transformation line/*prune* allele are shown (Figure 2E). Rescue of the prune eye phenotype by the transgene in question is evident by obtaining male progeny with wild-type eye coloration.

The eye color of transformants containing the *pHS-PN*$^+$*4* transgene remained prune in color even after several heat pulses. The fact that this truncated protein is stable and abundant after one 1-hr heat pulse (Figure 6, lane 6) argues that the *pHS-PN*$^+$*4* version of Prune is not functional.

Rescue of the prune eye color phenotype is observed for *pHS-PN*$^+$*3* transgenes only and is observed even in the absence of a heat pulse. The eye color of *prune* flies containing the *pHS-PN*$^+$*3* insertion appears completely wild type. The coloration of the eye does not alter when the animals are given several heat pulses and this type of ectopic overexpression of Prune protein does not appear deleterious to the animals.

Features of the Prune protein: The predicted amino acid sequence of Prune has some noteworthy features (Figure 3). FROLOV *et al.* (1994) described a putative transmembrane region of 17 amino acids present early in the second exon (amino acids 51–67). This region (indicated in Figure 3 by brackets) lacks features typical of a signal sequence for a type I transmembrane protein (VON HEINE 1986), and also does not conform precisely to the conventions of type II transmembrane proteins. The hydrophobic domains in type II transmembrane proteins typically initiate at positions between residues 29 and 88 and range from 19 to 25 residues in length (LANDRY 1991). The Prune hydrophobic region is initiated within this region of the protein, yet is somewhat shorter than most type II hydrophobic regions. The hydrophobic domains in type II transmembrane proteins are typically preceded by one or more basic residues and have several glycine or proline turn-inducing residues within them. The hydrophobic domain of Prune is immediately preceded by acidic residues (three within a stretch of 11 amino acids) and the nearest basic residue is 12 amino acids upstream. KYTE and DOOLITTLE's (1982) hydropathy analysis of the Prune protein gives this region a value of 2, which is no higher than four other shorter hydrophobic stretches within Prune (Figure 4). Typical transmembrane sequences have hydropathy values of 3 or more according to this analytical method. In addition, this stretch of hydrophobic amino acids has some hallmarks of an amphipathic helix. For these reasons, we infer that Prune is not a transmembrane protein.

The positions of the cysteines in Prune have an interesting pattern (Figure 3). The first five cysteines are regularly spaced and three more cysteine residues reside near the carboxy terminus. The spacing of CYS and HIS residues at the carboxy-terminus resembles that of a Zn-finger motif, albeit with a rather long "finger" between the CYS and HIS pairs (STRUHL 1989).



C

INSERTION		PROGENY ♀ #	♀ EYE COLOR	♂ #	♂ EYE COLOR
pHS-PN+3A	(0 heat shocks)	85	WT	83	WT
pHS-PN+4B	(5 heat shocks)	471	WT	0	–
pHS-PN+4B	(4 heat shocks)	65	WT	0	–
pHS-PN+4B	(3 heat shocks)	184	WT	0	–
pHS-PN+4B	(1 heat shock)	295	WT	0	–
pHS-PN+4B	(0 heat shocks)	56	WT	0	–

E

INSERTION		PROGENY ♀ #	♀ EYE COLOR	♂ #	♂ EYE COLOR
pHS-PN+3A	(0 heat shocks)	108	WT	94	WT
pHS-PN+4B	(5 heat shocks)	165	WT	156	PN
pHS-PN+4B	(4 heat shocks)	110	WT	98	PN
pHS-PN+4B	(3 heat shocks)	65	WT	57	PN
pHS-PN+4B	(1 heat shock)	55	WT	46	PN
pHS-PN+4B	(0 heat shocks)	80	WT	75	PN

FIGURE 2.—Tests for rescue of *prune/Killer of prune* lethality and the prune eye color phenotype by two putative Prune ORFs. (A) Transformation plasmids used to transform *y* w^{67C}; *ca* awd^{Kpn} flies. *pHS-PN*$^{+}$*3* contains the longer version of Prune ORF (Figure 1). *pHS-PN*$^{+}$*4* is an amino-truncated version of *pHS-PN*$^{+}$*3*. Both constructs contain *P*-element ends necessary for insertion into genomic DNA and the *miniwhite* gene for scoring for the presence of the insertions. (B) Test Cross 1 tests for *prune/Killer of prune* lethality. No male progeny will be produced from this cross unless functional Prune protein is expressed. *y w; ca* awd^{Kpn} males homozygous for the transformation plasmid on the second chromosome were tested. The insertion site of the transformation plasmid varies from line to line. (C) Progeny recovered from Test Cross 1. The number and sex of the progeny were scored after the indicated number of 1-hr heat shocks *per diem.* The results for only one test cross per transformation plasmid/*prune* allele combination are shown. All other combinations gave similar results. (D) Test cross 2 tests for the ability of *pHS-PN*$^{+}$*3* or *pHS-PN*$^{+}$*4* to rescue the prune eye color phenotype. The female progeny in this cross will have wild-type eye color; the male progeny should have prune eye color in the absence of functional Prune protein. Only second chromosome *pHS-PN*$^{+}$*4* insertion stocks (B, D, and F) were tested in Test Cross 2. All four *pHS-PN*$^{+}$*3* transformation lines had inserts on the second chromosome. (E) Progeny recovered from Test Cross 2. The number, sex, eye color, and number of heat shocks given are indicated. The results for only one test cross per transformation plasmid/*prune* allele combination are shown. All other combinations gave similar results.

Production of anti-Prune antibody: A Prune expression vector was prepared and Prune protein was purified as described. The final purified Prune protein was analyzed by SDS PAGE (Figure 5). The insoluble nature of the protein made purification difficult. We consider the end product obtained to be significantly enriched for Prune protein, but not "pure". The enriched protein was eluted from a SDS 10% polyacrylamide gel before injecting into rabbits, and rabbit anti-Prune antibody was purified as described.

The affinity-purified anti-Prune antibody recognizes the bacterially overexpressed Drosophila Prune protein

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                                                               ▽
ATG TGC TTT CTA CGA TTT TTG GCC CAG GCC AGG GGC ACC TTG GGA CGG CAT CTG GCG GAG
M   Ⓒ   F   L   R   F   L   A   Q   A   R   G   T   L   G   R   H   L   A   E    20

GCC TCA CCA GTT GCC TGG GCT GCT GCT CCC GAC GTT TCC GGC CGG AAA TTA CAT CTG GTA
A   S   P   V   A   W   A   A   A   P   D   V   S   G   R   K   L   H   L   V    40

ATG GGC AAC GAA TCG TGT GAC TTG GAC TCC[GCC GTT TCG GCC GTC ACT TTG GCT TTT GTC
M   G   N   E   S   Ⓒ   D   L   D   S   A   V   S   A   V   T   L   A   F   V    60
    ▼ (pn77C33)
TAC GCG CAG CGT CAT CGG GAG]CAC GAC TAT GTA CCT ATA CTG AAC ATT CCT CGC CGG GAC
Y   A   Q   R   H   R   E   H   D   Y   V   P   I   L   N   I   P   R   R   D    80
                                ▼ (pn38)
TAC CCG TTG AAA ACC GAG GTG GGC CAC TTG TTT GTG AAA TGT GGG ATT GCC GAG CCC GTG
Y   P   L   K   T   E   V   G   H   L   F   V   K   Ⓒ   G   I   A   E   P   V    100

TTG CTC TTC CGA GAC GAT ATT CCC CGG GAA GTG GTC CAG GAT GTG AAC GTT ATT CTC GTG
L   L   F   R   D   D   I   P   N   D   V   V   Q   D   V   N   V   I   L   V    120

GAC CAC CAT GTA AGC CCG CTG GCG CCA AAT GTT ACT GAA ATT TTG GAT CAC AGG CCC TTG
E   H   H   V   S   P   L   A   P   N   V   T   E   I   L   D   H   R   P   L    140
                                                    TAC (pnA)
GAG GAC AGC AGT CCA TCC TTC AAG CAG CTG CCA ACA CTC TGC CAA CTG GAC ATA GAT GCC
E   D   S   S   P   S   F   K   Q   L   P   T   L   Ⓒ   Q   L   D   I   D   A    160
                TTC (pnA)
TCG GTG GGT TCC TGC GCC ACT CTG GTG GCC CAG CGG TAT TTG GCA GAG GAC CAA CCC CGA
S   V   G   S   Ⓒ   A   T   L   V   A   Q   R   Y   L   A   E   D   Q   P   R    180
                    TAG (pn18a)
TCC ACT AGC GTG GCC CAG CTG CTG CAC GCC ACC ATC GTG CTG GAC ACA ATT AAT TTT GCA
S   T   S   V   A   Q   L   L   H   A   T   I   V   L   D   T   I   N   F   A    200

CCC GCG GCC AAG CGC TAC GGG CCA AAG GAC GAA GCC ATG GTA CAG AAG TTG GAG AGC GAG
P   A   A   K   R   Y   G   P   K   D   E   A   M   V   Q   K   L   E   S   E    220
          (pn12C)
CTT AAC CGT AAG GAC GCT CAA AGA AGT AGC CTT TTT GAT GAG CTA GTG GCT GCA AGG GCG
L   N   R   K   D   A   Q   R   S   S   L   F   D   E   L   V   A   A   R   A    240

GAT ATT AGT AAG CTA ACT CTC ACC GAA GTT TTG CGC AAG GAT ATG AAG GTC TTG CAA ACC
D   I   S   K   L   T   L   T   E   V   L   R   K   D   M   K   V   L   Q   T    260

GAT CGT CAG GTG GTT CCC TTA GCT GGA ATG CCC ATC CTA GTC AGA GAT TTT GTG GAG AAA
D   R   Q   V   V   P   L   A   G   M   P   I   L   V   R   D   F   V   E   K    280
                                    ▼ (pnpw2)
AGC GGC GCC GAA AAA GCC GTT CGC GAG TTT GGC GTG GAG AGT AAC CTT TTG GTT ATC CTG
S   G   A   E   K   A   V   R   E   F   G   V   E   S   N   L   L   V   I   L    300

GGA ATG TAT GTA TCA CCT GCC GAT GGC CAG GTG CAG CGT GAC CTG GCC TTG ATC TCT CTC
G   M   Y   V   S   P   A   D   G   Q   V   Q   R   D   L   A   L   I   S   L    320

TCC GGC CAA GGC CAA TTC GTT CAA CGC GTC CAG CAA GCA CTG ATG GAG TCT AAC GAT CCA
S   G   Q   G   Q   F   V   Q   R   V   Q   Q   A   L   M   E   S   N   D   P    340

AAA TTG GAG TTG CGA CCT CAC GAG GTG GAC ACC CGC TTT ATG GGC GGC TGC TTC TTG CGC
K   L   E   L   R   P   H   E   V   D   T   R   F   M   G   G   Ⓒ   F   L   R    360
                   ▼ (pn2)
CAA CAC AAC GTC CAG GCC ACC AGA AAG CAC ATC CTG CCC ATT GTT AAG CGA GCG CTG CTT
Q   H   N   V   Q   A   T   R   K   H   I   L   P   I   V   K   R   A   L   L    380

GAA TGG GAA GCG GAT CAC GCC TGC GAT TGT GAC GAG GTG TAC TTC TTC AAG GAG AAG CCG
E   W   E   A   D   H   A   Ⓒ   D   Ⓒ   D   E   V   Y   F   F   K   E   K   P    400

CAG CTG GGA CTC TCT TAA
Q   L   G   L   S   *
```

FIGURE 3.—Prune protein sequence. Regions of sequencing discrepancy are double underlined. The longest hydrophobic region is bracketed. The intron position is denoted by ▽. Cysteine residues are circled. The position of the inserted elements in *prune* alleles *prune*^{77C33}, *prune*^{38}, *prune*^{1}, *prune*^{2}, *prune*^{3}, and *prune*^{pw2} are indicated by ▼. The 7-bp deletion in *prune*^{12C} is boxed. The base substitutions in *prune*^{A} and *prune*^{18a} are indicated above the line of sequence.

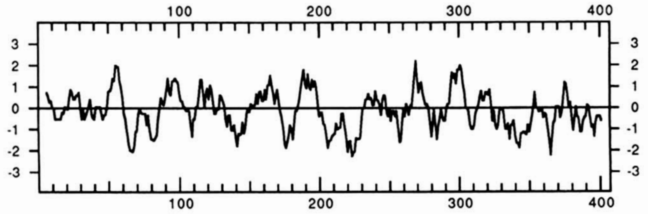

FIGURE 4.—Kyte and Doolittle hydropathy plot of the conceptual prune protein using a window size of 11.

on Western blots and shows some cross reactivity with other bacterial proteins (Figure 6, lanes 1–3). The antibody also recognizes both the full length and amino-truncated forms of Drosophila Prune overexpressed in Drosophila (Figure 6, lanes 6 and 8) in addition to other higher and lower molecular weight Drosophila proteins, but does not recognize Drosophila proteins with similar size to Prune; therefore, this antibody preparation is useful for Western blot analysis. The small amount of Prune-sized protein in uninduced lanes (Figure 6, lanes 4, 5, and 7) is derived from the endogenous *prune* gene product that is present in these wild-type transgenic third instar larvae in small quantity (Figure 8, lane 7). In addition, the antibody recognized the endogenous Prune protein (Figure 6, lanes 9–12) expressed in pupae. This stage coincides with the appearance of drosopterin pigments in the eye and Prune protein was expected to be abundant at this stage. The level of Prune protein expression does not appear to be drastically altered in animals homozygous for *yellow, claret, white, awd*^{Kpn}, *ebony, red,* or *multiple wing hairs,* which are some of the phenotypic markers used to maintain the *pHS-PN*$^+$ transformation vector and *awd*^{Kpn} stocks.

The size of the full-length Prune protein recognized by the anti-Prune antibody is the same for all the different forms of Prune: Prune expressed from cDNA in bacteria, Prune expressed from cDNA in Drosophila, or Prune expressed from the endogenous *prune* gene in Drosophila (Figure 6). This is an indication that the cloned cDNA contains the entire Prune coding region. However, the band recognized by the anti-Prune antibody is larger than the size predicted from the amino acid sequence (44.5 kD). On our SDS 10% polyacrylamide gels, the Prune band migrates with the 50-kD marker (both prestained and unstained markers were used in this analysis). As expected, the amino-truncated form of Prune expressed from *pHS-PN*$^+$*4* migrates to a lower position than the full length forms on all the gels we have run. Therefore, we believe that the apparent larger size of the Prune protein in some of our gel analyses is due to anomalous migration in our SDS-PAGE system for reasons we cannot explain. In sample extracts that contain large quantities of Prune protein, a smaller protein band also appears (Figure 6, lane 2, 3, and 8) that we interpret to be a degradation product. The higher and lower molecular weight bands were presumably generated from contaminating bacterial proteins in the "purified" Prune protein preparation.

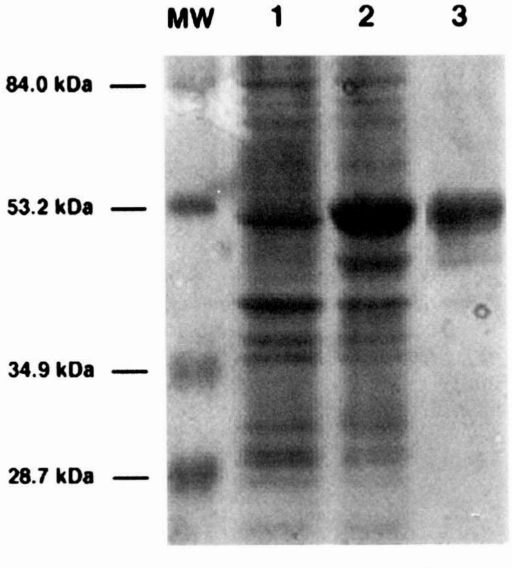

FIGURE 5.—Prune protein expression. Each lane contained 20 μg protein. Lane 1, IPTG-induced *BL21* bacterial cell extract; lane 2, *BL21* cells expressing Prune; lane 3, purified Prune protein.

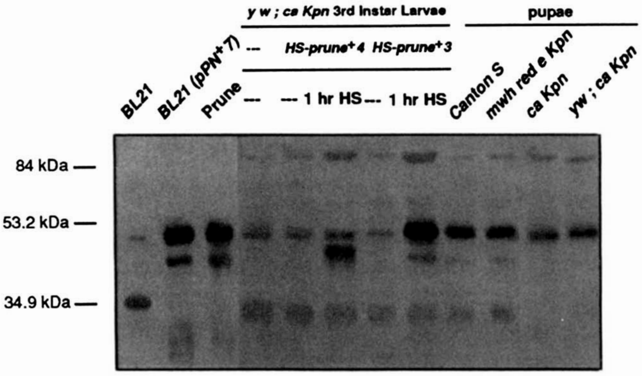

FIGURE 6.—Purification of anti-Prune antibody. Lanes 1–3 demonstrate the specifity of anti-Prune antibody for Drosophila Prune protein expressed in bacterial cells. Lane 1, *BL21* cell extract, 20 μg total protein; lane 2, extract of *BL21* cells expressing Prune protein, 0.5 μg total protein; lane 3, purified Prune protein, 0.05 μg. Lanes 4–8 demonstrate the specifity of the antibody for Prune protein expressed in *y w; ca Kpn* larval extracts. Lane 4, control larval extract; lanes 5 and 6, larvae harboring *pHS-PN*$^+$*4* transgene; lanes 7 and 8, larvae harboring *pHS-PN*$^+$*3* transgene. Larvae in lanes 6 and 8 were heat shocked for 1 hr at 37°. Lanes 9–12 are extracts of wild-type pupae and pupae with *awd*^{Kpn} in different genetic backgrounds, 20 μg each lane.

Analysis of *prune* mutants: We analyzed the DNA sequences of nine *prune* mutants *prune*^{77C33}, *prune*^{38}, *prune*^{A}, *prune*^{18a}, *prune*^{12C}, *prune*^{pw2}, *prune*^{1}, *prune*^{2}, and *prune*^{3} (Figure 3). *prune*^{77C33} contains a *P* element inserted within codon 62, which provides an in-frame stop codon four amino acids into the inserted sequence. The resulting conceptual mutant protein would thus be 65 amino acids in length. *prune*^{38} contains a *P*-element insertion in the second exon. The first two codons of the insertion in frame with the Prune sequence are stop codons and the resulting conceptual mutant protein

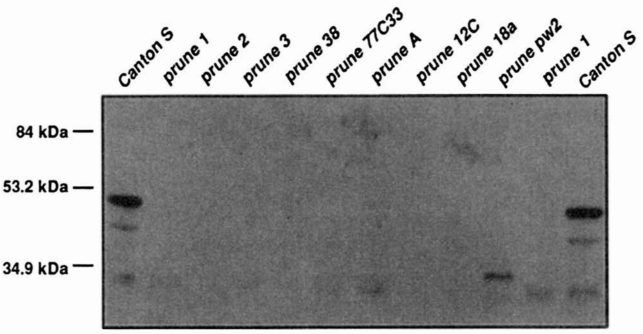

FIGURE 7.—*prune* mutant Western. Twenty micrograms late pupal extract of each *prune* allele was analyzed.

would thus be truncated to 88 amino acids. *prune*^{A} is an interesting mutant because it encodes a full-length protein with two amino acid substitutions. The first is a CYS to TYR substitution at amino acid 154. The second is a SER to PHE substitution at amino acid 164 (Figures 3 and 4). The mutation in *prune*^{18a} is a single base pair substitution of T for C within codon 186. This results in a stop codon at this position. *prune*^{12C} has an 8-bp deletion of nucleotides 665–672 that introduces a frame shift. The sequences downstream of this deletion produce four novel amino acids then a stop codon. *prune*^{12C} would thus be expected to encode a 225-amino acid protein that is wild type in sequence except for the final four amino acids. *prune*^{pw2} contains a *P* element inserted into codon 290 that provides an in-frame stop codon four amino acids into the inserted sequence. The resulting conceptual mutant protein would thus be 294 amino acids in length. *prune*^{2} contains an insertion of AAA G after nucleotide 1092 that throws the sequences out of frame introducing 11 novel amino acids and then a stop codon. The conceptual mutant *prune*^{2} protein would thus be a 375-amino acid protein that is wild type in sequence except for the final 11 amino acids. The mutation found in a stock labeled *prune; stw; kar e* was found to be the same mutation as in *prune*^{2}. The remaining two mutants, *prune*^{1} and *prune*^{2}, contain insertions within the *prune* coding region. This was observed by Southern blot analysis of the mutants using the *prune* coding region as probe and by an increase in size of a PCR-generated DNA fragment derived from the *prune*^{1} and *prune*^{2} protein coding regions (data not shown). In addition to the mutated sequences, we have noted two nucleotide polymorphisms that occured together in *Canton S* wild-type strains at codon 353 (TTT changed to TTC) and 359 (TTG changed to CTG). Both these sequence changes are silent polymorphisms.

Figure 8 is a Western blot of pupal extracts of several homozygous *prune* mutants. None of the mutants except *prune*^{pw2} accumulate Prune protein. *prune*^{pw2} expresses Prune protein to an appreciable level, yet the protein is truncated. Thus carboxy-terminal truncations of the Prune protein are nonfunctional. We have already demonstrated that amino-terminal deletions of Prune protein are nonfunctional (*pHS-PN*$^{+}$*4* expression). The fact that no Prune protein is seen in other *prune* mutants suggests that these mutant proteins are unstable, but does not rule out that their RNAs are unstable or untranslatable.

Previous reports describing the transcript sizes of some *prune* mutants as analyzed by Northern blot have indicated that *prune*^{2} produces a wild-type size transcript, *prune*^{pw2} and *prune*^{3} produce a truncated transcript, and *prune*^{38} and *prune*^{1} produce a larger-than-wild-type transcript. In addition, the *prune*^{1} chromosome was demonstrated to contain a mobile *422* element in the 2E genomic region (TENG *et al.* 1991; FROLOV *et al.* 1994). Our Western blot, Southern blot, and sequence analyses of these mutants are consistent with these observations.

Developmental profile of Prune expression: No drosopterin pigment is detectable before eye pigment formation, the accumulation of drosopterin pigments peaks during later pupal stages (12–13 days at 20°) (EVANS and HOWELLS 1978). Maximum accumulation of Prune protein precedes drosopterin pigment formation and the appearance of any pigments in the eye (9–10 days at 20°, Figure 8, lane 9). A small amount of Prune protein is present at most stages, and Prune protein accumulates to maximum levels during pupal and adult development.

The Prune protein present in the embryonic stages could be maternal deposition of Prune protein and/or from early zygotic transcription. *prune/Killer of prune* animals with no functional *prune* gene and no maternal supply at *prune* products live until the late third instar stage. In addition, genetically *prune/Killer of prune* animals that have received maternal supplies of both *awd*^{Kpn} and *prune* products also survive until late third instar (STURTEVANT 1956; HACKSTEIN 1971; unpublished observations, this lab). Therefore the maternal supply of *prune* product is protective against the lethal effects of *awd*^{Kpn} until the maternal supply of *prune* product is depleted. It is this absence of functional *prune* product in the presence of Awd^{Kpn}, which eventually causes death of the entire organism, and a very small amount of Prune protein is required to prevent this death (Figure 2).

The developmental Western data supports this interpretation. During mid-third instar, the animals have the least amount of Prune protein (Figure 8), and this is slightly before the onset of *awd*^{Kpn} transcription and also slightly before *prune/Killer of prune* lethality. It is interesting to note that maximal accumulation of both Awd and Prune occur during later stages of development, with Awd accumulation peaking slightly before Prune accumulation (third instar *vs.* early pupae).

Homology to yeast exopolyphosphatase: A BLAST database search of proteins related to Prune (ALTSCHUL *et al.* 1990) reveals similarity to yeast exopolyphosphatase protein (WURST and KORNBERG 1994; WURST *et al.* 1995). The BLAST homology outlines five conserved

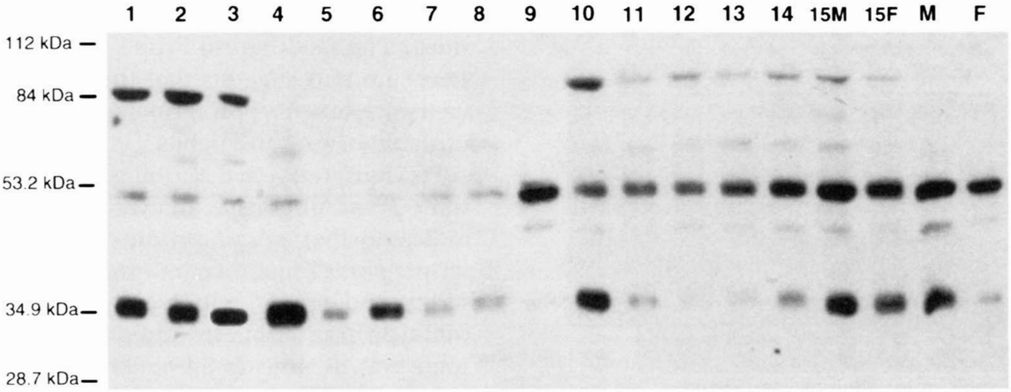

FIGURE 8.—Developmental Western of Prune protein expression. Forty micrograms of protein from each extract was analyzed. *Canton S* animals were collected at ~24-hr intervals; the lower lane number values represent younger animals. 1, embryos; 2, first instar; 3, early second instar; 4, late second instar; 5, early third instar; 6, mid-third instar; 7, late/wandering third instar; 8, larvae just beginning to secrete cuticle; 9, early white, sessile pupae; 10, early pupae, yellow pupal case; 11, yellow pupae, discs differentiated; 12, pupae just beginning to produce eye pigments, light brown; 13, pupae with dark red eyes, no wing coloration; 14, pupae with slight wing and thorax pigmentation; 15, pupae ready to eclose, separated by sex; 16, adult flies ~5 days after eclosion.

regions between these proteins. Overall, the similarity between the two proteins is low (23% identity, 41% similarity); however, the five conserved regions map to similar positions within each protein. Since no structure/function studies of yeast exopolyphosphatase have been performed, nor have any mutations been characterized, it is not possible to ascribe a particular function to these domains of similarity.

DISCUSSION

Sequence of *prune*: The DNA sequence of *prune* reported by TENG *et al.* (1991) and FROLOV *et al.* (1994) have three regions that differ (Figure 1). The reported amino acid sequence also differs between Region II and Region III because the sequence of TENG *et al.* (1991) contains two extra bases in Region II in comparison to the sequence of FROLOV *et al.* (1994) in Region II. The sequence of region III in TENG *et al.* (1991) has one more base than that of FROLOV *et al.* (1994); therefore, the protein sequence from Region III to the end of the protein is in agreement for both versions. The sequence of Region II (Figure 1) proved the most difficult to interpret due to a compression, while the sequence of Region III was easily read, and our sequence of both regions matches that of TENG *et al.* (1991). Since our sequence data from Region III is unambiguous in its interpretation, we are confident that the sequence in Region II matches that of TENG *et al.* (1991). We note that the reported sequences of Region II and Region III must be taken together [Region II of TENG *et al.* (1991) with Region III of TENG *et al.* (1991) or Region II of FROLOV *et al.* (1994) with Region III of FROLOV *et al.* (1994)], otherwise a stop codon would be introduced shortly after Region III. As an additional test for the correct amino acid sequence of the Prune protein, we analyzed the codon usage within both reported proteins in the 186-amino acid region of discrepancy. Codon usage tables from 341,043 nucleotides from Drosophila coding sequences have been compiled (MICHAEL ASHBURNER, personal communication) that provide data on preferred codons used by Drosophila. An analysis of the 186-amino acid region of discrepancy predicted by FROLOV *et al.* (1994) reveals that 14/186 of the predicted codons are most preferred codons. Similar analysis of the TENG *et al.* (1991) region reveals that 29/186 of the predicted codons are most preferred codons. The fact that twice as many of the codons between Region II and Region III from the TENG *et al.* (1991) version of Prune are preferred codons in comparison to the codon usage from the FROLOV *et al.* (1994) version of Prune lends further support for our interpretation of the sequenced regions.

Regions of Prune required for function: We have used germline transformation to demonstrate that a *prune* coding region of the length described in FROLOV *et al.* (1994) is functional by virtue of its ability to rescue both the prune eye phenotype and *prune/Killer of prune* lethality. Additionally, a 7.2-kb genomic DNA fragment has been shown by germline transformation to be sufficient to rescue both the eye color and lethal interaction with awd^{Kpn} (B. RUSKIN, D. TENG and T. VENKATESH, personal communication). The amount of Prune protein required to rescue both these phenotypes is very small: enough Prune is produced from an uninduced HSP70 promoter to rescue both phenotypes. The fact that such a small amount of protein can rescue both phenotypes suggests that Prune is an enzyme or a regulator of an enzyme, rather than a structural protein.

Functionally important domains of a protein can sometimes be revealed when mutations in these domains are introduced. In an attempt to identify functionally important domains in Prune, we analyzed the DNA sequences of several *prune* mutant alleles. Unfortunately all the mutants we analyzed failed to accumulate detectable protein by Western blot analysis of mutant pupae except one, *prune*^{pw2}. Prune^{pw2} is a carboxy-terminal truncated protein 290 amino acids in length. It is puzzling to note that the mutation in *prune*^{2} predicts a protein of 365 amino acids, 75 amino acids longer than Prune^{pw2}, yet this mutant protein fails to accumulate to an apprecible level. In addition, we have produced a stably expressed Prune protein missing 40 amino acids at the amino terminus (expressed from the *pHS-PN*$^{+}$*4* transgene) that is also nonfunctional. Our results indicate that the amino terminal 40 amino acids and the carboxy terminal 115 amino acids of Prune are required for function and the carboxy terminal 40 amino acids may be important for stability of the protein.

Overexpression of Prune is not deleterious: Wild-type Prune protein overexpressed from the heat-inducible hsp70 promoter in a wild-type background is not deleterious to the organism, nor does overexpressed Prune protein affect the color of the eye nor produce any additional phenotypes. The presence of Kpn protein in animals overexpressing Prune protein also does not alter the phenotype.

Is *prune* an essential gene? Since the accumulation of drosopterin pigments is reduced in *prune* mutants, not eliminated altogether, it is intriguing to speculate that *prune* mutations might be hypomorphs. If so, a mechanistic model that might also explain *prune/Killer of prune* lethality can be proposed. This model depends on the presumptions that *prune* is a vital gene, that all known *prune* alleles must then be hypomorphs, and that this reduction of Prune activity results in prune-colored eyes. According to this model, in lethal *prune/Killer of prune* animals, the function of the neomorphic NDP kinase subunit Kpn would be to further reduce the activity of Prune, and it is this severe reduction or elimination of an essential activity that would eventually cause death to the animal. However, the wide variety of mutations we have identified in *prune* alleles (insertions early in the protein coding region, a stop codon introduced half-way into the protein coding region, etc.), the fact that most of these mutations fail to accumulate protein as analyzed by Western blot, and the fact that none of these mutations is lethal suggests that *prune* is not an essential gene and that this model is not correct. Accumulated evidence suggests that *prune/Killer of prune* lethality is caused by the loss of Prune function and the gain of function of the Kpn protein. Our results are consistent with this hypothesis.

Prune has similarity to yeast exopolyphosphatase: Inorganic linear polyphosphates are abundant in the vacuoles of *Saccharomyces cerevisiae*, yet the function of polyphosphates in Saccharomyces has not been determined. In an attempt to understand the function of polyphosphates in yeast, several enzymes using polyphosphate as a substrate have been identified (KORNBERG 1995). Yeast exopolyphosphatase preferably utilizes polyphosphates of 250 residues in length and degrades them to inorganic phosphate.

The limited amount of Prune homology to yeast exopolyphosphatase may imply that Prune, like Awd, is involved in phosphatase/kinase reactions. While Prune may not encode a classic expolyphosphatase, it is intriguing to speculate that Prune may function as a phosphatase or kinase in a pathway that also includes Awd/Kpn. This is not untoward speculation since some of the intermediates in pteridine biosynthesis as well as some pteridine cofactors themselves contain phosphate groups. In addition, some of the biosynthetic enzymes in the pteridine pathway are also phosphorylated.

GTP is the initial substrate in the biosynthesis of pteridine eye pigments in Drosophila (FAN and BROWN 1976; MACKAY and O'DONNELL 1983). The final reaction in the production of GTP, addition of phosphate onto GDP, is catalyzed by Awd. The conversion of GTP to drosopterin pigments proceeds through a pathway that includes dihydroneopterin triphosphate (WIEDERRECHT *et al.* 1981; WIEDERRECHT and BROWN 1984). The phosphates are removed from dihydroneopterin triphosphate as a tripolyphosphate in a reaction catalyzed by the *purple* gene product (SWITCHENKO and BROWN 1985). Dihydroneopterin triphosphate is a precursor occupying a pivotal role in the biosynthesis of drosopterins and other eye pigments, the essential cofactor tetrahydrobiopterin, and other pteridine compounds. Thus is it not difficult to imagine how a perturbation in this pathway might elicit cellular responses that eventually cause death of the animal. The nature of this perturbation, the lethal focus of the *prune/Killer of prune* interaction, the precise function of Prune, and the cause of *prune/Killer of prune* lethality are unanswered questions currently under investigation.

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