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The sluggish-A gene of *Drosophila melanogaster* is expressed in the nervous system and encodes proline oxidase, a mitochondrial enzyme involved in glutamate biosynthesis

(phototaxis/behavioral mutant/cDNA sequence/transgenic organism/proline dehydrogenase)

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ABSTRACT **Certain gene mutations in *Drosophila melanogaster* cause sluggish motor activity. We have localized the transcription unit of the sluggish-A gene to a 14.7-kb region at the base of the X chromosome and have cloned corresponding cDNAs. The predicted protein product has significant sequence similarity to *Saccharomyces cerevisiae* proline oxidase (EC 1.5.99.8), a mitochondrial enzyme which catalyzes the first step in the conversion of proline to glutamate. In the mutant fly, mitochondrial proline oxidase activity is reduced and has kinetic properties different from those of the wild type, providing further evidence that the gene encodes proline oxidase. Indeed, the free proline level in mutant flies is elevated. When the mutant is rescued by transformation, the proline oxidase and free proline levels, as well as the motor and phototactic behavior, are restored to normal. During embryonic development the sluggish-A transcript is predominantly expressed in the nervous system. Significantly, it has previously been reported that a mouse mutant, PRO/Re, which has reduced proline oxidase activity and elevated free proline levels, also exhibits sluggish behavior.**

The systematic isolation of mutants of *Drosophila melanogaster* which exhibit specific alterations in behavior was pioneered over two decades ago using a countercurrent apparatus (1). The countercurrent method allows for the fractionation of a population of individuals on the basis of responses to various stimuli such as gravity, phototaxis, and locomotor activity (2). The method has been remarkably successful in revealing mutants involved in a diverse array of neural and neuromuscular processes, including signal transduction, neuronal connectivity, and cellular differentiation. Among the genes uncovered by the countercurrent method that have subsequently been cloned are no receptor potential-A (phospholipase C) (3), sevenless (a tyrosine kinase) (4, 5), technical knockout (a ribosomal protein) (6), small optic lobes (which contains a calpain-homologous domain) (7), retinal degeneration B (8), optomotor blind (9), and no on transient-A (10).

Here we characterize at a molecular level the sluggish-A (*slgA*) mutant,‖ which emanated from one of the original X chromosomal screens for phototaxis mutants. It has a normal electroretinogram and no gross neuroanatomical defects (11, 12).

MATERIALS AND METHODS

Mutagenesis and Mapping. To isolate behavioral mutants, wild-type (Canton-S) males were treated with ethyl methanesulfonate and their progeny were tested by the countercurrent method (1). The mutations were mapped relative to the markers yellow (*y*), white (*w*), diminutive (*dm*), chocolate (*cho*), crossveinless (*cv*), cut (*ct*), vermillion (*v*), miniature (*m*), garnet (*g*), forked (*f*), and a wild-type allele of yellow (y^+) transposed to the short arm of the X chromosome (13) (Fig. 1 *Left*).

Behavioral Assays. *Phototactic behavior.* The performance of flies in a countercurrent apparatus (1) was measured using a cycle time of 30 s and five transfers. Ten to 100 flies aged between 3 and 7 days were tested at a time.

Locomotor activity. Individual flies were tested in a 10 × 10 × 1-cm gridded, cardboard-and-glass chamber (15) after settling for 60 s. Movement from one square into another scored 1 activity point. Movements were monitored for 60 s. Ten flies of each genotype were tested individually, and the results were averaged. In another test for locomotor activity a 0.6 × 70-cm glass tube was used. Single flies were transferred to the tube by means of an aspirator and shaken to the midpoint. The tube was placed on a white surface under overhead fluorescent light, and the number of times a fly crossed the centimeter markings on a ruler was measured for five consecutive 60-s intervals.

Molecular Protocols. DNA and RNA extraction, blotting, and hybridization were performed as outlined (7). cDNA clones were isolated from an adult head library in λgt11 (16) by using restriction fragments from the genomic landscape shown in Fig. 2. Probes from the proximal 12 kb yielded 12 clones from 60,000 screened. Inserts from 8 of these were subcloned into pEMBL8+ (17). The longest cDNA (3.3 kb), as well as shorter cDNAs, was gel purified and sonicated, and fragments of 0.3–1.0 kb were cloned into the *Sma* I site of M13mp10. M13 subclones were sequenced with Sequenase (United States Biochemical), and compressions were resolved with dITP. Sequences were assembled and analyzed with Staden and Genetics Computer Group software, and databases were searched with FASTA (18). Alignments were scored with ALIGN (19).

Germ-Line Transformation and *in Situ* Hybridization. Construct pGL1 consists of a 7.2-kb *Eco*RI–*Nhe* I fragment ligated into the *Eco*RI and *Xba* I sites of pW8 (20). pGL2 contains a 14.7-kb *Eco*RI–*Xho* I fragment in pW8. It was made by first cloning a 3.5-kb *Eco*RI–*Xba* I fragment into the *Eco*RI and *Xba* I sites of pW8. An 11.2-kb *Xho* I–*Xba* I fragment was then cloned into the *Xho* I and *Xba* I sites of this plasmid. pGL1 and pGL2 were injected into white embryos as described (7). Male flies carrying a single autosomal

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L07330).

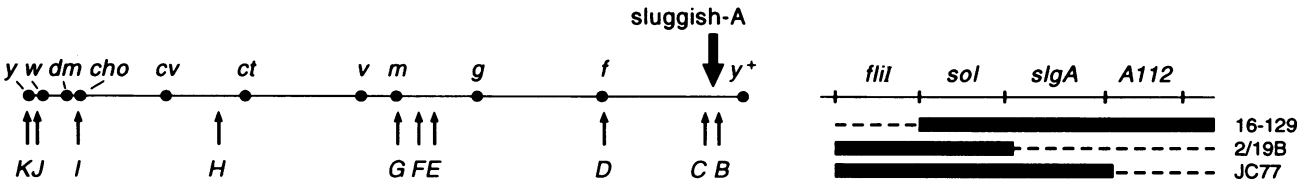

FIG. 1. Mapping of sluggish mutations. (*Left*) Map positions of the sluggish mutations (*slgA* to *slgK*, marked A to K) in relation to morphological mutations of known position on the X chromosome (13). All the sluggish mutations are completely recessive, except *slgK*, which is partly so. (*Right*) Localization of the *slgA* region by use of chromosomal rearrangements and known genetic complementation groups (12, 14). Deficiencies are shown by dotted lines, and normal chromosomal sequences by black bars.

insertion of pGL1 or pGL2 were crossed to homozygous *w slgA* females. *In situ* hybridization on whole embryos was performed essentially as described (21) except that digoxigenin-labeled DNA probes were prepared by using single primers and a modified thermal cycling reaction.

Proline Oxidase Assay. Mitochondria were prepared from batches of ≈500 male flies by a modification of the method of Tupper and Tedeschi (22). Flies were chilled (0°C) and homogenized in 0.25 M sucrose/1 mM EDTA/50 mM potassium phosphate buffer, pH 7.5, with 15 strokes of a homogenizer with a Teflon pestle. After centrifugation at 200 × *g* for 10 min, the upper two-thirds of the supernatant was recovered and centrifuged at 6000 × *g* for 10 min. The mitochondrial pellet was resuspended in the homogenization medium by gentle pipeting. All steps were carried out at 0–4°C. Proline oxidase activity was measured essentially as described (23). Reactions were conducted in a volume of 1 ml containing 75 mM potassium phosphate buffer (pH 7.5), various concentrations of L-proline, and 20 μl of mitochondrial suspension. The tubes were shaken at 37°C and 300 rpm for 30 min. Then, 1 ml of 0.5% *o*-aminobenzaldehyde/5% trichloroacetic acid in ethanol was added, and the tubes were incubated at room temperature for 20 min. After centrifugation, the absorbance at 443 nm of the supernatant was read against a blank treated identically except that proline was omitted. Activity was calculated by using a molar absorption coefficient of 2710 $M^{-1} \cdot cm^{-1}$, with 1 unit of activity corresponding to 1 $nmol \cdot min^{-1}$. Protein was measured by a modified Lowry procedure (24) with bovine serum albumin as standard.

Proline Assay. Groups of five flies were homogenized in 200 μl of redistilled methanol. After centrifugation, the supernatants were evaporated under vacuum, and the residues were dissolved in AminoQuant buffer A and analyzed on a Hewlett-Packard AminoQuant 1090M system (25).

RESULTS

Genetics of sluggish Genes. The map positions of the 11 sluggish loci uncovered in the phototaxis screen (denoted *slgA* to *slgK*) are shown in Fig. 1 *Left*. The locations, and the markers used as references, are (from left to right) *slgK*, 0.3 ± 0.05 relative to *y* and *w*; *slgJ*, 1.15 ± 0.1 relative to *y* and *w*; *slgI*, 5.0 ± 0.4 relative to *y* and *cho*, also shown to be at the right of *dm* (4.6); *slgH*, 19.0 ± 0.25 relative to *cv* and *v*, also shown to be at the left of *ct* (20.0); *slgG*, 36.4 ± 0.3 relative to *m* and *g*, 36.6 ± 0.3 relative to *v* and *f*; *slgF*, 38.6 ± 0.15 relative to *v* and *f*; *slgE*, 40.0 ± 0.25 relative to *v* and *f*; *slgD*, close to *f*; *slgC*, 66.8 ± 0.3 relative to *f* and a y^+ transposition at the right of the centromere; *slgA*, 67.7 ± 0.2 relative to *f* and y^+; and *slgB*, 68 ± 1 relative to *f* and y^+. Since 11 loci were uncovered on the X chromosome (only 2 of which had more than one allele), it can be estimated from the Poisson distribution that the genome contains a minimum of about 150 such loci. In this paper we focus on the only remaining mutant stock, sluggish-A, which we have localized between the lethal complementation groups *flil* and *A112* by using deficiencies that break in this genetically well-characterized region (Fig. 1 *Right*) (12, 14).

Molecular Analysis of the *slgA* Region. We have performed an extensive chromosomal walk and determined the molecular breakpoints of deficiencies *16-129, 2/19B* (7) and *JC77* (Fig. 2) which delimit the region containing the *slgA* gene. The genetic and molecular data together indicate that *slgA* is within the 28-kb region shown in Fig. 2. Three transcription units are detected when Northern blots of poly(A)$^+$ mRNA from embryonic, larval, pupal, and adult stages are probed with radiolabeled genomic DNA fragments from across the 28-kb region. Probes from the area of transcription unit 1 hybridize to a single transcript of 2.1 kb. Probes from the area of transcription unit 2 hybridize to a major transcript of 3.5 kb, with weaker bands at 2.2 and 6.0 kb (data not shown). The weaker bands may be alternatively spliced products from the same gene or may emanate from a different region of the genome. Probes from the area of transcription unit 3 hybridize to a 3.6-kb transcript, with much weaker hybridization to one of 2.5 kb (Fig. 3).

Rescue of the *slgA* Phenotype. To determine which of the three transcription units corresponds to *slgA*, transgenic organisms were constructed. Constructs pGL1 and pGL2, containing genomic DNA from the regions shown (Fig. 2), were placed into the germ line of w^- embryos by using a *P*-element vector carrying a w^+ marker (20). Seven independent transformants were recovered with pGL1, and three

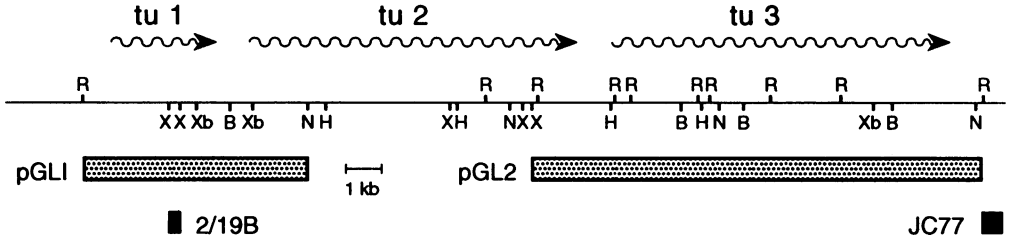

FIG. 2. Molecular map of the *slgA* region. The uncertainties in the extents of the two breakpoints which define this area molecularly are shown by solid boxes. Restriction sites for *Eco*RI (R), *Xho* I (X), *Xba* I (Xb), *Bam*HI (B), *Nhe* I (N), and *Hin*dIII (H) are as drawn. Wavy arrows indicate the three transcription units (tu 1, tu 2, and tu 3) in this region. The genomic DNA fragments which have been inserted into the transformation vector pW8 are denoted pGL1 and pGL2 and are shown by stippled boxes. Only pGL2 was capable of rescuing the *slgA* phenotype.

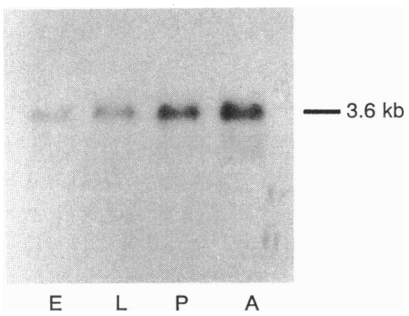

FIG. 3. Transcription analysis of the *slgA* region. A Northern blot of poly(A)$^+$ mRNA prepared from different developmental stages (E, embryo; L, larva; P, pupa; A, adult) was probed with radiolabeled *slgA* cDNA (a 1.2-kb *Bam*HI fragment from transcription unit 3). The amount of mRNA loaded in each lane was approximately equal as shown by reprobing the filters with a *D. melanogaster ras* gene which is uniformly expressed during development (data not shown).

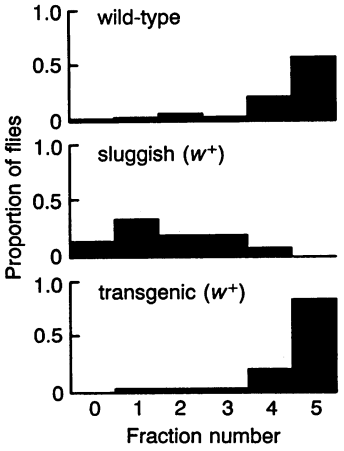

FIG. 4. Countercurrent distributions of Canton-S (wild-type), w^+ *slgA* mutant, and transgenic (w^+ *slgA*, pGL2) flies. Fraction number indicates the number of times flies moved toward the light source. Number of flies tested: Canton-S, 105; *slgA*, 109; transgenic, 111.

with pGL2. Transformants carrying a single autosomal insertion of pGL1 or pGL2 were crossed to *w slgA* flies. Transgenic flies (w^+) and nontransgenic full sibs (w^-), as well as the original mutant *slgA* (w^+) flies, were tested for their phototactic behavior and locomotor activity. Wild-type flies move rapidly toward light when excited, and hence they predominate in fraction 5, whereas *slgA* mutant flies move much more slowly and are distributed more uniformly (Fig. 4). It should be noted that both *slgA* (w^-) and *slgA* (w^+) flies behave similarly in all tests. Furthermore, the phototactic behavior of neither the Canton-S wild type nor the *slgA* mutant in a countercurrent apparatus has changed significantly since the last published test (compare Fig. 4 with figure 1 in ref. 11). The critical result is that transgenic individuals carrying a single copy of pGL2 in a *slgA* background are indistinguishable from wild type in their phototactic behavior (Fig. 4). Similar results were obtained with the other two independent pGL2 transgenic lines. pGL2 thus carries DNA sequences sufficient to rescue the *slgA* phenotype. On the other hand, a single copy of pGL1 in the *slgA* background fails to rescue the *slgA* phenotype. Since transcription unit 3 is the only complete transcription unit contained in pGL2, it must correspond to *slgA*. Northern and genomic Southern analyses on *slgA* mutant flies show no detectable changes from the wild-type patterns. Thus, the lesion is likely to be small, in keeping with the expected results of ethyl methanesulfonate mutagenesis, which mainly gives rise to point mutations.

Locomotor behavior was also tested in an activity chamber (15) and in a long glass tube. In the activity chamber, it was found that wild-type flies and transgenic individuals carrying a single copy of pGL2 moved with normal frequency (36 and 37 activity points, respectively), whereas *slgA* (w^-) mutant individuals and transgenic flies carrying pGL1 were very inactive (1.3 and 1.0 activity points, respectively). In the glass tube, transgenic flies carrying pGL2 moved almost as rapidly as control (w^+) individuals (32 and 37 cm/min, respectively) whereas full-sib *w slgA* mutant flies were far slower (6 cm/min).

To investigate whether the primary defect in the *slgA* mutant is more likely to be in the visual or locomotor systems, movement *away* from light was measured in a countercurrent apparatus. In this case, all nontransgenic and transgenic individuals stayed near the light source (data not shown). By mosaic fate mapping (26) experiments, we found that the focus of the *slgA* mutation—that is, the part of the adult fly where the mutation exerts its primary effects—maps to the thoracic ganglion, consistent with a defect in neural function.

We have also noted that the *slgA* fly, while walking after a mechanical shock, initially flutters its wings, a behavior that does not arise in normal flies even after repeated stimulation. Compared to a wild-type fly, its movement pattern can best be described as "indecisive," with multiple changes in direction. This behavioral characteristic is also restored to normal in transgenic flies.

Whole-Mount *in Situ* Hybridization. In embryos, transcription of the *slgA* locus, detected with a *slgA* cDNA probe, is first seen at the beginning of germ-band shortening, with staining of a small patch of cells corresponding to the anal pad (Fig. 5*A*). This is later followed by a metameric pattern of staining in the developing central nervous system (Fig. 5 *B* and *C*). Control embryos exhibit no specific staining (Fig. 5*D*).

DNA Sequence of *slgA*. Genomic DNA fragments from the area of transcription unit 3 (Fig. 2) were used to isolate adult head cDNAs (16). The longest cDNA, 3.3 kb, was sequenced on both strands and found to predict a protein of 669 amino acids (Fig. 6*A*). Part of the predicted protein has significant

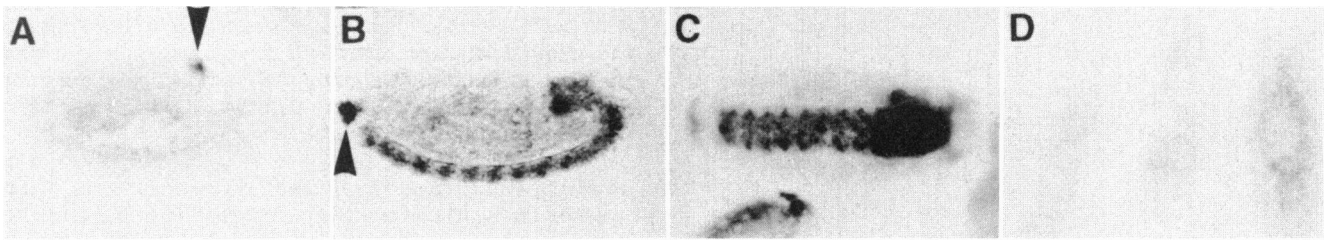

FIG. 5. *In situ* hybridization on whole *D. melanogaster* embryos with digoxigenin-labeled *slgA* cDNA probe. (*A*) Early embryogenesis, lateral view, antisense probe. (*B*) Mid-embryogenesis, lateral view, antisense probe. (*C*) Mid-embryogenesis, ventral view, antisense probe. (*D*) Embryos at different stages, sense probe. For *A–C*, anterior is to right. Arrowheads, patch of cells corresponding to the anal pad.

A

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1    MALLRSLSAQRTAISLVYGRNSSKSSNSVAVAACRSFHQRGNGSTSIAGEGAASESTRGVNGARFLHSGDRPLQASTLVQPEVVSSETVKRSMKQESSQE 100
101  KNPSPAGSPQRDPLDVSFNDPIAAFKSKTTGELIRAYLVYMICSSENLVEHNMTLMKWSKNVLGQRLFTLLMKATFYGHFVAGEDQIKIIPTLERLRSFG 200
201  VKPILDYSVEEDITQEEAEKREVESSVSSAGDKKEEGSMPQYHVDKSFADRRYKVSSARTYFYLNEATCERNMEIFIKCLEAVSGATFGTGITAIKLTAL 300
301  GRPQLLLQLSEVIMRTRKYMEDMVGGQGNVLTHHKTIKDLEKYYATLGDNKDVKEFLNNVTSDKEGILHLFPWSGIVDEDSQLSDTFRVPDPQTGQMRRL 400
401  ISQIPPKEEEMFRNMIRRLNTIVKAAADLDVRIMVDAEQTYFQPAISRITLEMMRKYNKDKAIVFNTYQCYLRETFREVNTDLEQAKRQNFYFGAKLVRG 500
501  AYMDQERDRAKSLGYPDPVNPTFEATTDMYHRTLSECLRRIKLMKDCDDDARKIGIMVASHNEDTVRFAIQQMKEIGISPEDKVICFGQLLGMCDYITFP 600
601  LGQAGYSAYKYIPYGPVEEVLPYLSRRAQENKGVLKKIKKEKRLLLSEIRRRLMRGQLFYKPKGNYVPI                                669
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B

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sigA TYQCYLRETFREVNTDLEQAKRQNFYFGAKLVRGAYMDQERDRAKSLGYPDPVNPTFEATTDMYHRTLSECLRRIKLMKDCDDDARKIG-IMVASHNEDT 565
     |:| |||:: :: :|: |: ::: :| |||||||:: |::|:: : : | :    :|:: |:|:::: :: : :   ::::: :| ::|||||| ::
PUT1 TWQLYLRDSGDHILHELKLAQENGYKLGLKLVRGAYIHSEKNRNQII-FGDKT-----GTDENYDRIITQVVNDLII----NGEDSYFGHLVVASHNYQS 381

sigA VRFAIQQMKEIGISPEDKV-ICFGQLLGMCDYITFPL--GQAGYSAYKYIPYGPVEEVLPYLSRRAQEN                                631
      ::::: :|:: :: :|  | :|||||| | :|: |  :::: :  ||:|:|| |:  || || |||
PUT1 QMLVTNLLKSTQDNSYAKSNIVLGQLLGMADNVTYDLITNHGAKNIIKYVPWGPPLETKDYLLRRLQEN                                450
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FIG. 6. (*A*) Amino acid sequence of the predicted sigA protein. (*B*) Alignment with part of the PUT1 proline oxidase of *S. cerevisiae*. Identical amino acids are indicated by vertical lines, and conservative substitutions by colons.

sequence similarity to the product of the *Saccharomyces cerevisiae* proline utilization gene *PUT1* (Fig. 6*B*) (27). The *PUT1* gene encodes mitochondrial proline oxidase (proline dehydrogenase). A number of shorter cDNAs were also examined by restriction analysis and DNA sequencing, and most were found to be truncated variants of the 3.3-kb cDNA.

Proline Oxidase Measurements. To test directly whether the *sigA* gene encodes proline oxidase, assays were performed on mitochondrial preparations. Initial measurements indicated that an L-proline concentration of 100 mM was above the apparent K_m of the wild-type proline oxidase. At this concentration, little difference was observed in the proline oxidase levels in mitochondria from various strains, with Canton-S (wild type), Amherst (wild type), and w^- giving values of 18.7, 16.2, and 19.9 units/mg, respectively. In contrast, in six separate experiments, the proline oxidase activity of the *sigA* mutant was reduced to 39 ± 7% of the wild-type activity. When 10 mM proline was used, the assay of the *sigA* mutant gave 17 ± 3% of wild type.

Assays were therefore performed over a range of proline concentrations, and the results were analyzed with Lineweaver–Burk plots. In these plots (Fig. 7), the proline oxidase in the mutant was strikingly abnormal, whereas that of transgenic flies carrying a single copy of pGL2 was close to wild type. Wild-type preparations gave linear plots from 10 to 400 mM proline, with a K_m for proline of ≈50 mM. Values in this vicinity have also been reported for the human enzyme (28). In contrast, the mutant extracts exhibited a curved Lineweaver–Burk plot over this range, with the activity falling more sharply at lower proline concentrations than expected from simple Michaelis–Menten kinetics (Fig. 7). We also observed that the activity in mutant extracts was less stable than wild type on overnight incubation at 0°C. The simplest explanation of these results is that the *sigA* mutant encodes a mutant proline oxidase of lower activity and lower stability than the wild-type enzyme. The nonlinearity of the Lineweaver–Burk plots could be due to stabilization of the mutant enzyme at higher proline concentrations.

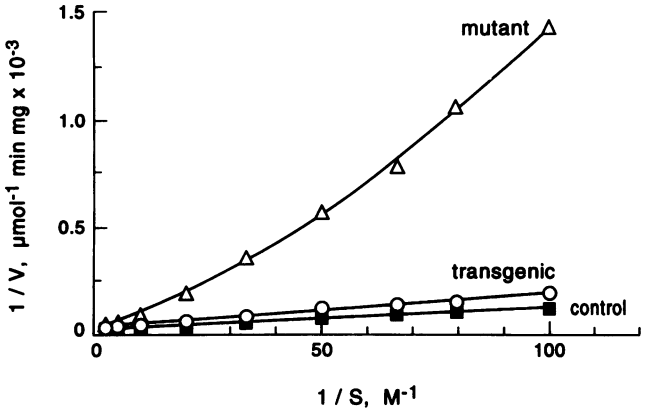

FIG. 7. Lineweaver–Burk plot of proline oxidase activity of mitochondrial preparations from different genotypes. ■, *white*; ○, transgenic individuals carrying a single copy of pGL2 in *w sigA* mutant background; △, *w sigA* mutant (full sibs to transgenic).

Proline Measurements. One anticipated consequence of a defective proline oxidase system would be an elevation of free proline levels (28, 29). We therefore measured the free amino acid levels in *w sigA* mutant, w^-, Canton-S, and transgenic flies carrying a single copy of pGL2 in the mutant background. Proline was the only amino acid altered, being consistently elevated an average of 2-fold in *w sigA* mutant flies relative to w^-, Canton-S, or transgenic flies (data not shown). It should be noted that the normal level of free proline in wild-type flies is substantial (5–10 nmol per fly).

DISCUSSION

Analysis of the amino acid sequence of the predicted sigA protein reveals that a region of 165 amino acids toward the C terminus exhibits 30% sequence identity and 69% similarity to part of the proline oxidase encoded by the *PUT1* gene of *S. cerevisiae* (27) (Fig. 6). This match is highly significant, giving a score of 13 SD, when compared with the results of 100 randomized runs using the program ALIGN (19). Although the predicted sigA protein is 669 amino acids in length while the yeast protein consists of only 476 amino acids, our direct biochemical data also indicate that the *sigA* locus encodes a protein with proline oxidase activity.

Proline oxidase is the first of two enzymes involved in the conversion of proline to glutamate in mitochondria. It and 1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12), the second enzyme, are both encoded by nuclear DNA, synthesized on cytoplasmic ribosomes, and then imported into mitochondria. In rat liver, proline oxidase is associated with the inner mitochondrial membrane (23), and in *E. coli* it is

associated with the plasma membrane (30). Based on its suggested identification as proline oxidase, the slgA protein would be expected to possess a mitochondrial import sequence. Examination of the N-terminal region of the predicted slgA protein reveals that the first 30 amino acids are rich in lysine and arginine, as well as serine and threonine, and are free of aspartic and glutamic acid. When plotted on a "helical wheel" the charged residues cluster on one side of the helix. These sequence characteristics are indeed typical of a mitochondrial import sequence (31).

At this point, the nature of the link between the defective proline oxidase in the *slgA* mutant and the behavioral phenotype is not clear. *In situ* hybridization shows that, in the developing embryo, the most abundant transcription is in the developing nervous system. The transcript continues to be strongly expressed in the adult (Fig. 3) although we have not determined the times and sites of transcription at that stage. In human type I hyperprolinemia, proline oxidase activity is reduced and in some cases there is associated mental retardation (28). Activity is also reduced in the PRO/Re mouse (29), with a concomitant increase in serum proline. Strikingly, it has been observed that "PRO/Re mice were sluggish in their movements from weaning onwards" (29), suggesting that significant parallels between the *slgA* mutant fly and the PRO/Re mouse may extend across the biochemical and behavioral levels. High proline levels can destroy pyramidal and granule cells in the rat hippocampus, and from this it has been argued that these excitotoxic effects may be related to the neurological and cognitive deficits associated with hyperprolinemia (32). Given that free proline levels are elevated in *slgA* mutant flies and PRO/Re mutant mice, a direct toxic effect of proline may contribute to the phenotype. Another possibility is that the *slgA* phenotype may result from impairment of the mitochondrial proline shuttle (33).

A deficiency in mitochondrial proline oxidase may affect glutamate levels in specific neural cell populations. Glutamate has been identified as the transmitter at neuromuscular junctions in *Drosophila*, so a deficiency in its level could possibly account for reduced motor activity. In higher organisms, where glutamate also functions as a neurotransmitter, the focus to date has largely been on the various glutamate receptor families. Glutamate is believed to play an important role in the neocortex and hippocampus and in neuroendocrine regulation in the hypothalamus. It is possible that proline oxidase plays a significant role in the formation of some neural glutamate pools (34). The possible role of proline as a neurotransmitter has recently become the focus of considerable attention, with the cloning of a proline transporter molecule (35, 36). This is expressed in or near glutamatergic neurons, whereas the relevant enzymes for synthesis of glutamate from proline are found in glial cells, not neurons (reviewed in ref. 35).

Finally, while *slgA* is the first mutant of its behavioral phenotype that we have analyzed in molecular detail, it is clear that many more mutants of similar character can be isolated. Furthermore, mutants isolated via the countercurrent method are already providing important clues, not only to the understanding of nervous system function in *Drosophila*, but also to mammalian systems, as originally foreshadowed by Miller and Benzer (37).

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