# The *tamas* Gene, Identified as a Mutation That Disrupts Larval Behavior in *Drosophila melanogaster*, Codes for the Mitochondrial DNA Polymerase Catalytic Subunit (*DNApol-γ125*)

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ABSTRACT

From a screen of pupal lethal lines of *Drosophila melanogaster* we identified a mutant strain that displayed a reproducible reduction in the larval response to light. Moreover, this mutant strain showed defects in the development of the adult visual system and failure to undergo behavioral changes characteristic of the wandering stage. The foraging third instar larvae remained in the food substrate for a prolonged period and died at or just before pupariation. Using a new assay for individual larval photobehavior we determined that the lack of response to light in these mutants was due to a primary deficit in locomotion. The mutation responsible for these phenotypes was mapped to the lethal complementation group *l(2)34Dc*, which we renamed *tamas* (translated from Sanskrit as "dark inertia"). Sequencing of mutant alleles demonstrated that *tamas* codes for the mitochondrial DNA polymerase catalytic subunit (*DNApol-γ125*).

INVERTEBRATE behavioral paradigms have been extensively and successfully used in the fruit fly *Drosophila melanogaster* (Heisenberg 1997) and the nematode *Caenorhabditis elegans* (Chalfie and Sulston 1981) in the search for genes involved in behavior. Vertebrate model systems have also benefited from a genetic approach to the study of behavior. Recently, a novel gene required for the generation of circadian rhythms was isolated in the mouse in a screen for semidominant mutations that disrupt circadian modulated locomotion (Vitaterna *et al.* 1994).

In Drosophila a genetic approach was fundamental to the identification of components of the phototransduction pathway underlying adult photobehavior (Heisenberg and Buchner 1977; Heisenberg and Wolf 1984; Koenig and Merriam 1977; Pak 1979; Zuker *et al.* 1985). In this case mutations were useful not only for the identification of gene products but also as instruments in the identification of specific cell types required for the performance of particular light-induced behaviors. For example, Drosophila mutant strains lacking different subsets of adult photoreceptors were used to address the role of these photoreceptor cell types in the performance of different types of photobehaviors. Photoreceptor cells R1 through R6, the outer photoreceptor cells, mediate optomotor responses while photoreceptor R7 is involved in fast phototaxis and some types of slow phototaxis (reviewed by Heisenberg and Wolf 1984).

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Traditionally, Drosophila genetic screens using behavioral paradigms have been conducted using adult flies. A few recent examples include the isolation of mutations that disrupt associative learning (Boynton and Tully 1992), circadian rhythms (Sehgal *et al.* 1992), and hygro- and/or thermosensation (Sayeed and Benzer 1996). Genetic screens using the third instar larva proved that this developmental stage is also a good model for the identification of novel behavioral genes (Kernan *et al.* 1994).

The Drosophila larvae are repelled by light during the foraging stage, which spans the beginning of the first instar until the onset of wandering behavior during the third instar stage. Once larvae leave the food substrate in search of an adequate site for metamorphosis, repulsion to light steadily decreases until soon before pupariation when the larva behaves indifferently toward a light stimulus (Sawin-McCormack *et al.* 1995).

Although the larval photoreceptor cell cluster has an organization somewhat similar to the adult compound eye ommatidium (Green *et al.* 1993), little is known about the functional organization of these cells. It has been reported that the larval photoreceptor cell clusters express the same rhodopsin genes as found in the adult compound eye (Pollock and Benzer 1988); however, it is not known whether the different rhodopsins are expressed in nonoverlapping sets of larval photoreceptor cells. Likewise little is known about how the two photoreceptor cell clusters modulate larval locomotion in response to light. The existence of two clusters suggests that modulation of locomotion may be triggered by the unequal stimulation of the two photosensitive organs (tropotaxis; Fraenkel and Gunn 1961). Alterna-

tively, the regular swinging motions of the anterior of the larva from left to right before the onset of locomotion may suggest a mechanism by which orientation relative to the light source is possible by comparing the light intensity at different points in time (klinotaxis; Fraenkel and Gunn 1961).

Here we report an investigation into larval photobehavior in Drosophila. A genetic screen was designed to identify mutations that disrupt the response of foraging third instar larva to light, from a collection of 64 second chromosome EMS-induced pupal lethals. The screen was based on a previously described population assay that we improved by supplementing the food substrate with vitamin A (Lilly and Carlson 1990; Sawin *et al.* 1994). We identified one mutant strain (*P183*), which showed reduction in the response to light of the foraging third instar larva in the population assay and no response in a single larva assay developed in our laboratory (Hassan *et al.* 1999). Deficiency and meiotic recombination mapping demonstrated that this mutation is allelic to mutations in a previously identified lethal complementation group, *l(2)34Dc* (Woodruff and Ashburner 1979). The *P183* strain displays disruption in the pattern of compound eye development and failure to undergo the behavioral changes characteristic of the wandering third instar larva. Further characterization of the behavioral phenotype demonstrated that the failure to respond to light was likely due to a locomotory deficit. Analysis of the sequence made available by the Berkeley *Drosophila* Genome Project (BDGP) together with the genetic mapping of the region strongly suggested that this gene [*l(2)34Dc*] encodes for the catalytic subunit of the mitochondrial DNA (mtDNA) polymerase. Sequencing of this gene from four available mutant alleles revealed changes consistent with the notion that this gene encodes for the catalytic subunit of the mitochondrial DNA polymerase. The gene represented by the *l(2)34Dc* complementation group was renamed *tamas* (*tam*), which in Sanskrit means "dark inertia."

## MATERIALS AND METHODS

**Stocks:** The lethal mutations and chromosome aberrations used in this study are described in FlyBase (FlyBase Consortium 1999) and listed in Table 1. We used an isogenized derivative of Oregon-R as a wild-type stock (kindly provided by Art Hilliker).

**Fly culture:** The flies were cultured in a medium that contained inactivated yeast, sucrose, 10% tegosept in ethanol, and acid mix (propionic acid and phosphoric acid) and was supplemented with β-carotene (1.25 g/liter). Crosses were set up with 5–10 pairs of flies in 100 × 25-mm glass vials and were scored for 9 days after the emergence of the first progeny. Prior to the behavioral screen, larvae were grown on media plates streaked with a thin layer of live yeast paste.

**Larval collection for behavioral assays:** The egg collection involved 2–3 precollections of 2 hr each on a fresh plate. The last 1-hr collection was retained and incubated for 80–85 hr at 25° before testing in the plate assay at between 80 and 90 hr after egg laying (AEL). For the pupal lethal screen we incubated the embryos for the first 36 hr at 30° so that the heterozygous flies carrying the *CyO, l(2)DTS513*$^{1}$ (*CyO, DTS*) chromosome would die. The plates with larvae were placed at 25° until 80–85 hr AEL. The balancer chromosomes in all stocks used for photobehavior experiments were exchanged for *CyO, Dp(1;2)y*$^{+}$ (*CyO-y*$^{+}$) and the X chromosome was substituted for one carrying the mutations $y^{1}$ and $w^{1}$, to facilitate selection of larvae based on the *y* (*yellow*) phenotype of the larval mouth hooks. For the individual larval assays, eggs were collected for 1 hr and incubated at 25°. At 20–22 hr AEL all newly hatched first instar larvae were cleared and after another 1-hr incubation period ~70 newly hatched first instar larvae were collected and transferred to a fresh food plate coated with yeast paste. Larvae were grown in a 12-hr L:D cycle. Third instar larvae were tested for photobehavior at 84 hr AEL, a minimum of 3 hr into the dark cycle.

**The plate assay:** The plate assay used in this study was similar to the one used by Lilly and Carlson (1990). Approximately 100 larvae were picked with a size 1 brush under safelight (20-W lamp with Kodak GBX-2 filter) and washed well with distilled water two or three times. The water was completely drained, and the larvae were collected and placed in the middle of the test plate (Fisher Scientific, Pittsburgh; 100 × 15 mm) layered with 15 ml of 1% agar. This plate rested on a light box with a template of dark and light quadrants between the light box and the plate. The larvae were allowed to distribute themselves with respect to their preference for light or dark quadrants. After 5 min the larvae in each quadrant were counted and a response index [RI = (dark − light)/(dark + light)] was calculated. Since the *DTS* lethality is leaky the final response index of the lines was recalculated after subtracting the number of escapers (surviving adults) from each quadrant in all tests (Figure 1).

**Optimization of the population plate assay:** The following experiments were carried out to improve larval response in the plate assay originally described by Lilly and Carlson (1990). Vitamin A deprivation in adult Drosophila has been shown to result in loss of mature opsin photopigments (Harris and Stark 1977). The lack of opsin reduces visual sensitivity probably due to a lack of functional opsin apoprotein or loss of opsin transcription itself (Harris and Stark 1977; Stark and White 1996). Supplementing our medium with vitamin A (β-carotene at 1.25 g/liter; Ashburner 1989) significantly increased the response index of wild-type strains. These results were confirmed by using vitamin A supplementation of Sang's minimal medium (kindly provided by William Stark; data not shown).

The relationship between light intensity and the response index was investigated using a series of neutral density filters placed between the plate and the light source (Hassan *et al.* 1999). We concluded that the Drosophila larva responds to different light intensities and that the light intensity used in our assays elicits maximum response.

**Checker assay:** This assay used a petri dish (100 × 15 mm; Fisher Scientific) containing 15 ml of 1% agarose. This dish is positioned over a checkered template of alternating 1-cm black squares. Dark squares blocked all light while the clear squares allowed transmission of light. The template and dish were positioned on a light box that had been modified to emit light only in a 11-cm-diameter area in the center of the box. During the test the template and dish were lit from below.

Individual larvae were removed from the culture dish with a moist paintbrush, rinsed with distilled water to remove any residual food particles, and placed in a pretest plate for a period of 1 min to allow them to acclimatize to the agar surface. They were then transferred to the center of the test dish. Larval movement was visualized using a Fujinon TV/

**TABLE 1**

**Fly stocks used in this study**

| Strains | Breakpoint/cytology | Genetic limits | Reference |
|---|---|---|---|
| | | 34D region | |
| *Df(2L)64j, Adh$^{n1}$L$^{2}$* | 34D-1-D2; 35B9-C1 | *l(2)34Db-l(2)35cF* | Ashburner *et al.* (1982) |
| *Df(2L)b87e25* | 34C1; 35C1 | *kuz-vas* | Alexandrov and Alexandrova (1991) |
| *Df(2L)b80c1,b80c1 noc$^{Sco}$* | 34D3;34E2 | *l(2)34Db-Ance* | Ashburner *et al.* (1982) |
| *In(2LR)b81a2* | 34D5;34D8;41D | *Sos+/−-tam* | Alexandrov and Alexandrova (1986) |
| *In(2L)b81l7* | 34D1-2;40F | *l(2)34Db-b* | Alexandrov and Alexandrova (1986) |
| *Df(2L)b81a2$^{L}$A80$^{R}$, cn bw* | 34D5;35A3.4;41D | *Sos+/−-pu* | This study |
| *Df(2L)b84a9, pr pk cn sp* | Not visible | *Sos-l(2)34Dg* | This study |
| *In(2L)b82c44* | 34D4-5;40h | *Sos-b* | Alexandrov and Alexandrova (1986) |
| *Df(2L)b85b2* | Not visible | *b-tam* | Alexandrov and Alexandrova (1986) |
| *Df(2L)b88b42* | Not visible | *b-tam* | Alexandrov and Alexandrova (1991) |
| *In(2L)b83b22* | 34D4-5;35C1-2 | *b-tam* | Alexandrov and Alexandrova (1986) |
| *In(2L)b79h1A* | 34D4;34D8;40E | *l(2)34Db-tam +/−* | Alexandrov and Alexandrova (1991) |
| *b tam$^{2}$ Adh$^{n4}$* | | | Woodruff and Ashburner (1979) |
| *b tam$^{3}$ Adh$^{n4}$* | | | Woodruff and Ashburner (1979) |
| *b tam$^{4}$ Adh$^{n2}$ pr cn* | | | Woodruff and Ashburner (1979) |
| *tam$^{9}$* | | | This study |
| *kuz$^{3}$* | | | Unpublished data |
| *l(2)34Db$^{3}$ b$^{1}$ Adh$^{n2}$ pr$^{1}$ cn$^{1}$* | | | Woodruff and Ashburner (1979) |
| *l(2)Sop2$^{1}$ Adh$^{D}$ pr$^{1}$ cn$^{1}$* | | | Gubb *et al.* (1984) |
| *In(2LR)Gla, Gla l(2)34De$^{2}$* | | | Woodruff and Ashburner (1979) |
| *b l(2)34Df$^{2}$ el rd$^{s}$ pr cn* | | | Alexandrov and Alexandrova (1996) |
| *b l(2)34Dg$^{1}$ Adh$^{n4}$* | | | Ashburner *et al.* (1982) |
| *b Sos$^{34Ea-6}$ Adh$^{n4}$* | | | Woodruff and Ashburner (1979) |
| | | 35B region | |
| *Df(2L)TE35BC-24, b pr pk cn sp* | 35b4-6;35E1-2 | | Gubb *et al.* (1984) |
| *In(2LR)Gla, Gla l(2)34De$^{2}$ l(2)35Bb$^{6}$* | | | Woodruff and Ashburner (1979) |
| *b Adh$^{D}$ l(2)35Bc$^{2}$ pr cn* | | | Woodruff and Ashburner (1979) |
| *b l(2)35Be$^{1}$ pr* | | | Woodruff and Ashburner (1979) |
| *b Adh$^{n4}$ l(2)35Bf$^{5}$* | | | Woodruff and Ashburner (1979) |
| *Adh$^{D}$ l(2)35Bg$^{2}$ pr cn* | | | O'Donnell *et al.* (1977) |
| *Adh$^{UF}$ Su(H)$^{8}$ cn* | | | Woodruff and Ashburner (1979) |
| | | Miscellaneous chromosomes | |
| *b pr c px sp* | | | |
| *Cyo, l(2)DTS513$^{1}$* | | | |
| *Cyo, Dp(1;2)y$^{+}$* | | | Mardahl *et al.* (1993) |
| *b Adh$^{n2}$ pr cn* | | | Grell *et al.* (1968) |
| *b Adh$^{n4}$ pr cn* | | | Grell *et al.* (1968) |

zoom lens (Fuji Optical Co.) attached to a CCD camera (Elmo Mfg. Co., TSE272S) and recorded on videotape (Fuji HQ-120, RCA VCR) until they reached the assay boundary or until 180 sec had elapsed. Larvae that failed to move out of the first square in 90 sec were not included in calculating the response index, as these animals may have been injured during preparation. Measurements of residence time were taken using the VCR timer and started 5 sec after the larva was placed in the center dark check. Response index [(time in dark square − time in clear square)/total time of test] was calculated on a per larva basis. The same assay was performed without the light stimulus (20-W lamp with Kodak GBX-2 filter) and a response index was calculated. Therefore two response indices were calculated per genotype. A response to light is represented by a significant effect of light on the response index (Hassan *et al.* 1999).

**Locomotory assay:** Locomotory assays were conducted under safelight (20-W lamp with Kodak GBX-2 filter). Larvae were rinsed and acclimated to the agar surface in a plate as described earlier and placed in the center of the test plate. After 5 sec recovery time, larval movement was videotaped for 30 sec. Tracings of larval path were obtained and scanned using a flatbed scanner (Apple Color OneScanner 600/27). Path length was measured using NIH-image software version 1.61b.

**Lethality mapping and complementation analysis:** One pupal lethal, *P183*, was identified from the screen. The lethality of this line was mapped by meiotic recombination using the multiply marked chromosome *black* (*b*), *purple* (*pr*), *curved* (*c*), *plexus* (*px*), *speck* (*sp*). Females of the genotype *P183/b pr c px sp* were crossed to *y$^{1}$ w$^{1}$; S/CyO-y$^{+}$* and putative recombinant chromosomes were recovered over *CyO-y$^{+}$*. Out of 367 lines tested 103 retained the *P183* lethality and only 1 of these carried *b*, suggesting that the *P183* lethality mapped close to

*b*. This recombinant was later determined to have been the result of a crossover between *b* and the lethal mutation located in 35B. Deficiency mapping was conducted using a set of deficiencies spanning the *black* region (see Figure 4 and Table 1 for a map of the region and the breakpoints of the deficiency stocks used). All deficiency chromosomes were kept over a *Cy* balancer. We tested for complementation by crossing the *P183*/*CyO-y*$^+$ flies to deficiency stocks. The presence of straight wing flies among the progeny of the cross was indicative of complementation of the *P183* chromosome by the deficiency. A minimum of 100 flies were counted for each cross. We determined that two nonoverlapping deficiencies spanning the 34D and 35B regions did not complement *P183* suggesting that *P183* carried two lethal mutations. These were mapped more precisely by crossing to other deletions and to alleles of known lethal complementation groups in these regions.

**Wandering test:** Bromophenol blue (J. T. Baker Inc., Phillipsburg, NJ; D293-01) was dissolved in the regular fly medium to the final concentration of 0.05%. The final egg collection was conducted in the bromophenol blue supplemented medium and the larvae were allowed to grow in the bromophenol supplemented medium. The larvae were removed at specified time points and photographed under a stereomicroscope (Zeiss, Thornwood, NY).

**Immunohistochemistry of the larval central nervous system:** Larval brains with the eye-antenna imaginal discs were dissected from third instar larvae in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (pH 7.4) for 45 min at room temperature. This was followed by three or four PBS washes. Brains were incubated for 1 hr in a blocking solution that contained PBS with 0.3% Triton X-100 and 5% goat serum at room temperature, followed by addition of a fresh 100 μl of blocking solution containing 2 μl of 24B10 primary antibody. After 8–12 hr of incubation at 4° the samples were washed with PBT for 4 hr, with changes every 10–20 min, incubated in a blocking solution as described above, and finally incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) for another 8–12 hr. Once again the specimens were washed thoroughly for 4 hr and stained with 0.5 ml of 3, 3′-diaminobenzidine (0.5 mg/ml; Sigma, St. Louis, 72H3614) in the presence of hydrogen peroxide. The reaction was stopped by washing several times with PBS. The samples were mounted in 70% glycerol in PBS and observed with a Zeiss axiophot microscope.

**Mitochondrial staining:** Staining to visualize mitochondrial mass was done using Mitotracker Red CMXRos (kindly provided by Molecular Probes Inc., Eugene, OR, M-7512) at 100 nm concentration made in PBS. The brains were dissected in PBS, incubated in the staining solution for 10 min at room temperature and mounted for observation using fluorescein filters.

**Environmental scanning electron microscopy of adult compound eyes:** We used environmental scanning electron microscope (ESEM) model 2020 manufactured by ElectroScan Corporation (now Philips ElectroScan). This technology does not require metal coating of the specimen that is to be visualized. Adult flies were etherized and placed on the ESEM mount. Flies were immobilized using water-based colloidal carbon glue for proper orientation. The electroscan was performed at 20.0 kV and 3.9 Torr.

**DNA sequencing:** Genomic DNA was extracted from 80–100 larvae of the appropriate genotype by selecting for the *yellow* mouth hook marker. The larvae were washed with distilled water and frozen in liquid nitrogen prior to grinding using a mortar and pestle. The powdered larvae were resuspended (10 μl/larvae) in homogenization buffer (0.1 m Tris HCl, pH 9.0; 0.1 m EDTA; 1% SDS) for 30 min at 70°. Fourteen microliters of 8 m KCl per 100 ml of homogenate was added and the homogenate was placed on ice for 30 min and then centrifuged at 15,000 rpm, at 4° for 15 min. The supernatant was precipitated with 0.5 volumes of isopropanol. The precipitate was centrifuged at room temperature for 5 min, washed with 500 μl of cold 70% absolute alcohol and again centrifuged at room temperature for 5 min at 15,000 rpm. The pellet was dried for 10 min at 37° and resuspended in 80–100 μl of sterile water for 8–10 hr at 37°. Templates for DNA sequencing were generated through PCR amplification using the following oligonucleotides: (A) 5′-CCCCACCAACTTCCATAATG-3′; 3′-GTTACTACTTCCCCTGGTCCA-5′; (B) 5′-GGTTGGACTTCAGTTGCCTTA-3′; 3′-CGTGTGGTGCAACAAAGTACT-5′; (C) 5′-GAGGAGTTACTACTTCCCCTG-3′; 3′-CTGTGGAGCTTAAGGATTCTG-5′; (D) 5′-TGTGGGTTCATCATTTTCATG-3′; 3′-ATCCCTAACAGCTACAGC-5′; (E) 5′-GGGCGTAAGTAGTCACAAACC-3′; 3′-AAGGAGACTTGGAGGCTGTTA-5′; (F) 5′-TAGAGGATGACGAAGAGCCGT-3′; 3′-GATGGAACCCACATGGATGAC-5′; (G) 5′-CAGCGATATGCAACTCCATAAC-3′; 3′-GTGGACTTCCTTCATCTGATG-5′.

The fragments generated by PCR amplification [using Platinum Taq DNA polymerase (GIBCO BRL, Gaithersburg, MD) in a GeneAmp 2400 machine (Perkin-Elmer, Norwalk, CT)] covered the whole gene including introns, beginning 354 bp upstream of the open reading frame start site and terminating 75 bp after the stop codon. The DNA fragments generated in the PCR reactions were purified using the QIAquick gel extraction kit (cat. no. 28704). Automated sequencing was done using the cycle sequencing protocol with Taq-FS enzyme and BigDye terminator chemistry in a Perkin-Elmer-ABI 373A Stretch machine. Each fragment was sequenced from at least two different PCR reactions from both directions. The progenitor strains of three of the *tam* alleles were used as controls for sequence alignments: *b Adh*$^{n4}$ for *tam*$^2$ and *tam*$^3$ and *b Adh*$^{n2}$ *pr cn* for *tam*$^4$ (see Table 1). The *tam*$^9$ mutation was confirmed by amplification of DNA using primer set B and primer set F, which provide overlapping sequence data ($n$ = 6). The mutation in *tam*$^2$ was identified by overlapping sequence data obtained using the primer set D ($n$ = 4). The mutation in *tam*$^3$ was identified using primer set E which also provides overlapping PCR product ($n$ = 4). The lesion in *tam*$^4$ was identified using the primers in set E ($n$ = 6). The sequence alignments were conducted using Clustal_X (Thompson *et al.* 1997) on a Power Macintosh computer.

## RESULTS

**Screen of a set of pupal or late larval lethal strains for disruption in photobehavior using the population plate assay:** The 64 EMS-induced second chromosome recessive lethal lines were tested in the plate assay using the dominant temperature-sensitive balancer *CyO*, *DTS*. The scheme is presented in Figure 1. In the first screen all lines were tested and those that showed a response index above 0.6 (20% larvae in the light and 80% in the dark) were discarded. The 11 lines that showed a response index below 0.6 were screened again and 2 lines retained that showed a response index of less than 0.5 (Figure 2). These lines (*P183* and *E22*) were then tested in a different genetic background using a *CyO-y*$^+$ balancer. The *E22* line was found to be a second instar lethal in this genetic background and was discarded. The *P183* line continued to show a response index below 0.5 (data not shown) and was further studied.

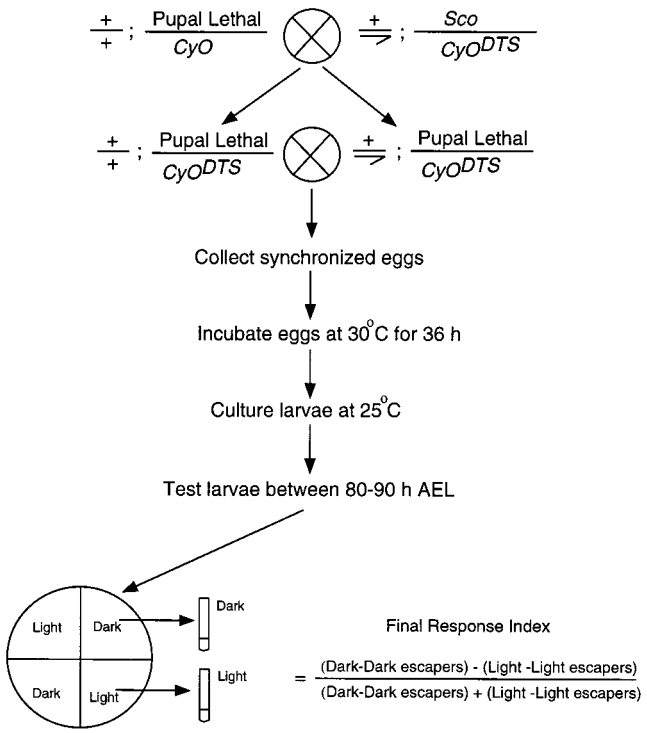



Figure 1.—The mutant screen.

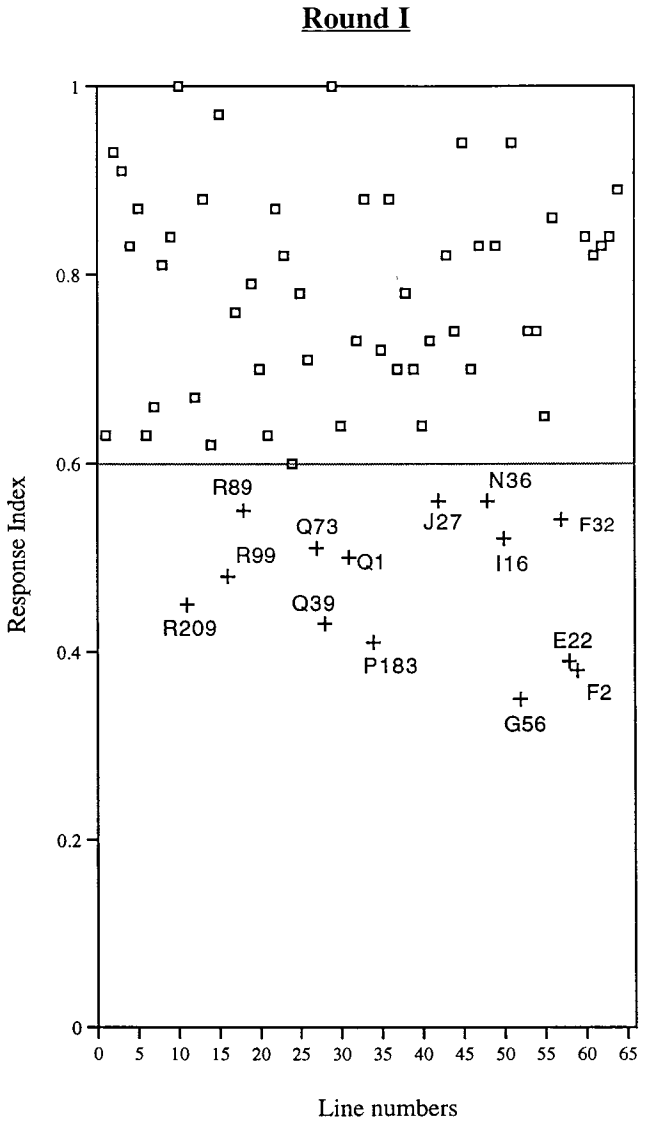
**Genetic analysis of *P183*: a double hit in the *l(2)34Dc* and *Suppressor of Hairless* genes:** Meiotic recombination mapping of the *P183* lethality was conducted using the *b pr c sp px* chromosome (see materials and methods for description). The results suggested that the lethality mapped very close to *b*. This region has been well characterized genetically and several lethal complementation groups have been identified (Woodruff and Ashburner 1979). We used a set of deficiencies that spanned the 34D-35C interval to map the *P183* lethality. A large deficiency in the region *Df(2L)64j* [Df(2L) 34D1-2;35B9-35C1] and two nonoverlapping deficiencies *Df(2L)b80c1* [Df(2L) 34D3;34D8-34E1] and *Df(2L)TE35BC-24* [Df(2L) 35B4-6;35E1-35E2] did not complement the *P183* lethality (Figure 3 and Table 2A). This suggested that *P183* carried at least two lethal hits in this region, one in the region 35B4;35C1 and the other located more distally at 34D. We crossed lethal alleles of the known complementation groups in these regions to *P183* (Figure 3; Table 2, B and C; Woodruff and Ashburner 1979). The lethality in the 35B region was found to be allelic to *Su(H)* mutations whose cytological map position is 35B9-10 (Figure 3; Table 2C; data not shown). The noncomplementing deficiency *Df(2L)b80c1* removes eight lethal complementation groups, *l(2)34Db-Ance* (Ashburner *et al.* 1982). All three available alleles of *l(2)34Dc* lethal complementation group did not complement the lethality of the *P183* strain, whereas alleles of the other lethals removed by *Df(2L)b80c1* complemented *P183* (Table 2B; data not shown).

Thus we concluded that the *P183* lethality was due to mutations in two genes, one in the 34D region [*l(2)34Dc*] and the other in the 35B region [*Su(H)*]. *Su(H)* alleles cause lethality during early pupal stages (Schweisguth and Posakony 1992). The gene represented by the lethal complementation group *l(2)34Dc* was renamed *tamas* (*tam*). Eight preexisting alleles of *l(2)34Dc* [$l(2)34Dc^{1-8}$] are listed by FlyBase (FlyBase Consortium 1999) and so we named the new allele $tam^9$ (Table 3). Additionally, a chromosomal inversion [*In (2L)b79h1A*; Figure 4] behaves as a hypomorphic allele of *tam* when heterozygous with other mutant alleles and was henceforth named $tam^{10}$ (Table 3). We separated $tam^9$ mutant allele from the $Su(H)^{P183}$ allele by recombination with a wild-type chromosome and checked for lethality against all three available *tam* alleles and chromosomal aberrations that uncover the *tam* gene (Table 4).

The $tam^9$ mutation causes lethality at the late third instar larval stage when heterozygous with a 34D deletion [*e.g.*, *Df(2L)b80c1*]. Two other alleles ($tam^2$ and $tam^4$) are early larval lethals when homozygous and one ($tam^3$) is a late embryonic lethal. $tam^2$, $tam^3$, and $tam^4$ lethalities are probably due to second-site mutations on these chromosomes because larvae carrying either one of these alleles and a deficiency also die at the late third instar stage (data not shown).

**The genetics of the 34D region:** The genetics of the 34D-35F region have been studied extensively by M. Ashburner and colleagues (*e.g.*, Woodruff and Ashburner 1979; Ashburner *et al.* 1982; Figure 3; Table 1). They have shown from deletion mapping that the lethal complementation groups in the immediate vicinity of *b* are *l(2)34Db*, *Son of sevenless* (*Sos*), *tam*, and *Sop2*: 75 out of 103 *b* deletions also remove these four loci (M. Ashburner and J. Roote, unpublished observations). That *l(2)34Db* is the most distal of these is shown by 4 proximally extending *b* deletions that remove *Sos*, *tam*, and *Sop2* but not *l(2)34Db* [*e.g.*, *Df(2L)b84a9*]. Both *Sos* and *tam* are adjacent to *b* by the criterion that many aberrations are either $Sos^-$, $b^-$, $l(2)34Dc^+$ or $Sos^+$, $b^-$, $l(2)34Dc^-$ [*e.g.*, *In(2L)b82c44* and *In(2L)b83b22*, respectively]. That *Sos* is distal and *tam* is proximal to *b* is confirmed by the aberration *In(2L)b81l7*, which is deleted for *l(2)34Db*, *Sos*, and *b*, but not *tam* or *Sop2*; therefore the genetic order is *l(2)34Db*, *Sos*, *b*, *tam*, *Sop2*.

**The *tam* gene product is required for the onset of wandering behavior in the third instar larva:** Drosophila larvae spend most of their time inside the food substrate (the foraging larval stage). During the third instar stage, in response to increased levels of ecdysone, they leave the food in search of an adequate site to pupariate and undergo metamorphosis (reviewed by Truman *et al.* 1994). This behavioral pattern is called wandering behavior and one characteristic aspect of the wandering phase is emptying of the gut as seen by the disappearance of blue-colored food (Maroni and Stamey 1983).



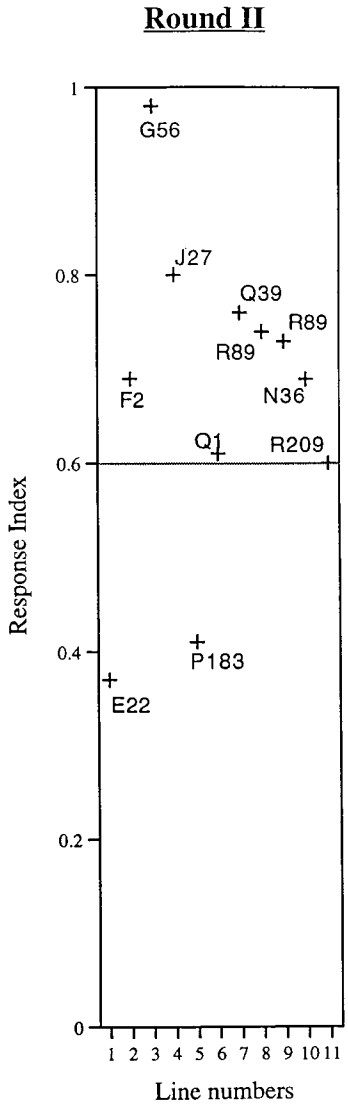




Figure 2.—Behavioral screen results. This is a scatterplot of the final response indices after the first round and the second round of screening. In the first round the lines that showed response index above 0.6 were discarded. Strains with response indices indicated by (+) were screened again. The second round resulted in two lines (*E22* and *P183*) considered to be putative behavior mutants.

Larvae homozygous for *P183* (double mutant) failed to wander out of the food substrate at the appropriate time. Consequently these larvae displayed a prolonged foraging phase that could last up to 20 days, after which they died. Prolonged foraging was visualized as larvae that retained bromophenol blue after the wild-type larvae had cleared their guts (Figure 4, A–D). This failure-to-wander phenotype was observed in larvae heterozygous for other alleles of *tam* and a deficiency of the 34D region (Figure 5, E and F and data not shown). Larvae homozygous for the recombinant $b^{-}$, $Su(H)^{P183}$ wander at the appropriate time and die at pupation (data not shown). Thus we conclude that the failure-to-wander phenotype is a consequence of loss of *tam* gene function.

**Mutations in the *tam* gene disrupt visual system development:** The isolation of mutations that affect photobehavior may also have important consequences for the study of pattern formation in the nervous system as mutations that ostensibly affect behavior may do so by disrupting developmental processes. To investigate the role of the *tam* gene in the larval response to light we analyzed the morphology of the visual system in *tam* mutant larvae with the 24B10 monoclonal antibody that labels a photoreceptor-specific antigen present in both the larval and developing adult visual systems in the third instar larva (Figure 5, A and B; Zipursky *et al.* 1984).

The visual system of the original double mutant strain *P183* was characterized by overall reduction in the size of the eye-antenna imaginal disc (Figure 5B). While labeling with the 24B10 monoclonal was found in the posterior portion of the disc, it was not seen in the usual clustering pattern representing the developing ommatidia. Rather, the expression of the epitope recognized by this monoclonal antibody seemed to be continuous throughout the developing retina. These observations may indicate that all cells in the disc were taking a photoreceptor fate. Subsequent analysis using the *P183* chromosome heterozygous with chromosomal deficiencies that remove either the 35B [*Su(H)*] or the

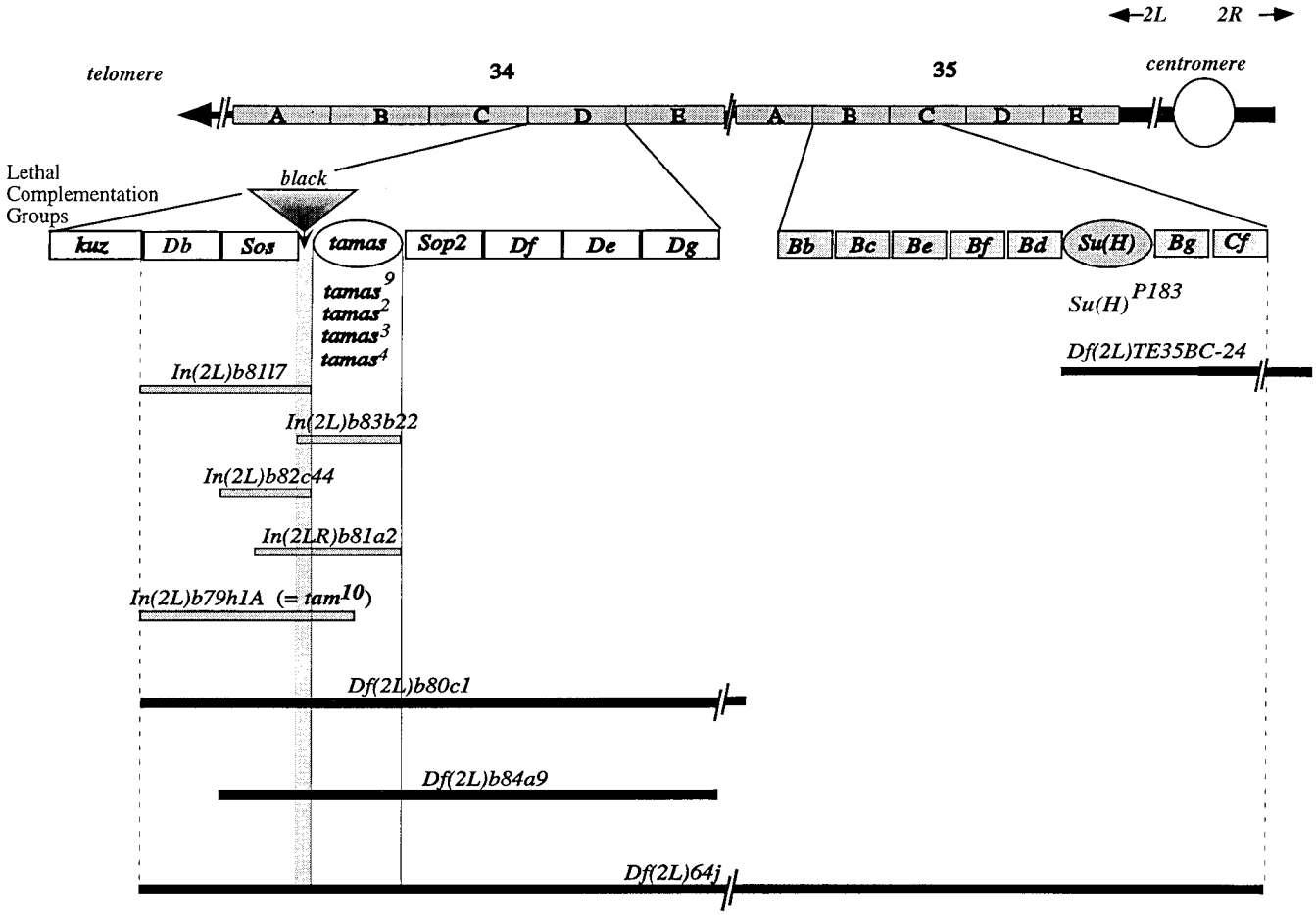



Figure 3.—Genetic analysis of *P183*. A diagram of the left arm of the second chromosome cytological regions, 34 and 35. The 34D and 35B regions are expanded to show the lethal complementation groups included in these subdivisions (Woodruff and Ashburner 1979). The rectangular boxes represent the lethal complementation groups, the oval-shaped boxes represent the lethal hits present in the *P183* line. The triangular insert in the 34D region is the nonvital gene *black*. Below the lethal complementation groups the chromosomal aberrations that were used in this study are shown. Solid bars depict the chromosomal segments that are deleted in the deficiency strains. In strains carrying chromosomal inversions the inverted segment is shown as a shaded bar.

34D (*tam*) regions and with mutant alleles of each gene demonstrated that this particular phenotype was due to disruption of the *Su(H)* gene (data not shown; Schweisguth and Posakony 1992).

Disruption of *tam* gene function led to less severe disruption in the pattern of the developing ommatidia by comparison with the original double mutant strain (Figure 5, C and D). The eye disc, while still reduced compared to a wild-type control, showed the usual pattern of clustering albeit somewhat disorganized. The projection of the photoreceptor axons into the optic lobes was also disrupted probably due to the abnormal differentiation of the retinal cells. Closer inspection of the eye disc under higher magnification suggested that older retinal cells were degenerating or losing the antigen recognized by the 24B10 monoclonal antibody (Figure 6F). The larval visual system was present; however, the projection defects observed in the developing adult visual system masked the terminus of the larval visual system, at that time in development. An interesting aspect of the phenotype of the visual system of *tam* mutant larvae was the delayed onset of retinal differentiation. 24B10 labeling could only be seen in *tam* mutant larvae several days after it was seen in wild-type larvae (Figure 5). The severity of these defects varies possibly due to the delayed onset of retinal differentiation.

The phenotype observed in the developing eye disc was reflected in the compound eye of adult escaper flies heterozygous for all lethal alleles of *tam* and a hypomorphic allele carried by *In(2L)b79h1A* (Table 3). These escapers have rough compound eyes that are significantly smaller than the control (Figure 6).

In *tam* mutant larvae there was an overall reduction in the volume of both the CNS and the imaginal discs reflected externally by the smaller than average larvae (Figure 4). The general morphology of the CNS as seen by labeling with the anti-ELAV monoclonal antibody was relatively normal (data not shown). The imaginal

**TABLE 2**

**Mapping lethality in the *P183* line**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| A. | | *Df(2L)TE35BC-24* | | *Df(2L)b80c1* | | *Df(2L)64j* | | | | |
| | *P183* | −(0/180) | | −(0/129) | | −(0/182) | | | | |
| B. | | *kuz*$^{3}$ | *l(2)34Db*$^{3}$ | *Sos*$^{6}$ | *tam*$^{3}$ | *Sop2*$^{1}$ | *l(2)34De*$^{2}$ | *l(2)34Df*$^{2}$ | *l(2)34Dg*$^{1}$ | |
| | *P183* | +(48/149) | +34/105) | +(38/120) | −(0/176) | +(47/132) | +(44/110) | +(32/123) | +(49/145) | |
| C. | | *l(2)35Bb*$^{5}$ | *l(2)35Bc*$^{2}$ | *l(2)35Be*$^{1}$ | *l(2)35Bf*$^{5}$ | *l(2)35Bd*$^{4}$ | *Su(H)*$^{8}$ | *l(2)35Bg*$^{2}$ | | |
| | *P183* | +(40/126) | +(65/209) | +(41/147) | +(38/117) | +(50/135) | −(0/262) | +(39/118) | | |

Minus indicates lethality and plus indicates viability. Numerator indicates the number of viable adult flies that were heterozygous either for the chromosomal aberration or the alleles of lethal complementation groups, with the *P183* chromosome. The denominator indicates the number of flies with a marked balancer chromosome. (A) Indicates mapping lethality using deficiencies in the *black* region. (B and C) Results of complementation test using representative alleles of lethal complementation groups in the 34D region and 35B region, respectively.

discs, seen under Nomarski microscopy, were reduced by comparison to wild-type controls but the overall morphology was apparently normal (data not shown).

**The reduced response to light of *tam* mutant larvae is due to a locomotory deficit:** The disruption in the response to light was reevaluated using an individual larval assay (Hassan *et al.* 1999). This assay measures the response to light in individual larvae as the relationship between residence time in light and dark squares with or without the light stimulus (see materials and methods for details). Larvae homozygous for the original double mutant chromosome *P183* or for the recombinant strain carrying only the *tam*$^{9}$ mutation showed no response to light in the Checker assay (data not shown; Figure 7). Larvae heterozygous for *tam*$^{9}$ and other mutant alleles of the *tam* gene responded to light (*tam*$^{3}$, Figure 7; *tam*$^{2}$, data not shown) suggesting partial complementation of this phenotype.

Visual inspection of the larvae during the course of the Checker assay suggested that these organisms moved considerably less than the wild-type background strain. In fact, measurements of the distance traveled in 30 sec in the absence of a light stimulus confirmed these observations (Figure 8). *tam*$^{9}$/*Df(2L)b80c1* or *tam*$^{3}$/*Df(2L)b80c1* larvae could not be tested in the Checker assay because of a severe deficit in locomotion (Figures 7 and 8). This deficit in locomotion was not seen in *tam*$^{9}$/+, *tam*$^{3}$/+, or *Df(2L)b80c1*/+ larvae (Figure 8) and these mutant larvae responded to light in the Checker assay (Figure 7). Larvae carrying the heteroallelic combination *tam*$^{9}$/*tam*$^{3}$ moved significantly less than the control larvae *tam*$^{3}$/+ ($P = 0.005$) and appeared to show a similar trend when compared to *tam*$^{9}$/+ larvae ($P = 0.099$; Figure 8). The measure of locomotion of *tam*$^{3}$/+ and *tam*$^{9}$/+ showed no statistically significant difference (Figure 8). We concluded that lack of response to light seen in the *tam*$^{9}$ mutant larvae was due to a primary deficit in locomotion that is uncovered by *Df(2L)b80c1* and that this allele behaves as a hypomorph.

***tam* codes for the catalytic subunit of the mitochondrial DNA polymerase (*DNApol-γ125*):** The genetic location of *tam* suggested that this gene was included in the sequence recently released by the Berkeley *Drosophila* Genome Project (S. Misra, personal communication). The superimposition of the genetic map onto the physical map showed that *tam* was located on the P1 DS00941 in a region tightly packed with open reading frames coding for known gene products (Ashburner *et al.* 1999; Figure 9). The region of interest was delimited distally by *Sos* (*BG:DS00941.4*), which codes for guanine nucleotide exchange factor (Simon *et al.* 1991; Bonfini *et al.* 1992), and proximally by the profilin associated protein gene, *Sop2* (*BG:DS00941.7*), transformants of which have been shown to rescue the lethality of the *l(2)34Dd* complementation group (Hudson and Cooley 1998; Figure 9). Since the glutamate decarboxylase

**TABLE 3**

**Interallelic complementation among *tam* alleles**

| | $tam^9$ | $tam^2$ | $tam^3$ | $tam^4$ |
|---|---|---|---|---|
| $tam^2$ | −(0/154) | | | |
| $tam^3$ | −(0/396) | −(0/302) | | |
| $tam^4$ | −(0.171) | −(0/167) | −(0/295) | |
| $tam^{10}$ | −/+(121/412) | −/+(22/562) | −/+(69/427) | −/+(6/67) |

Minus indicates lethality and −/+ indicates reduced viability. Numerator indicates the number of viable adult flies that were heterozygous for *tam* alleles and the denominator indicates the number of flies with a marked balancer chromosome. New nomenclature of *tam* alleles: $tam^2$ corresponds to $l(2)34Dc^2$, $tam^3$ corresponds to $l(2)34Dc^3$, $tam^4$ corresponds to $l(2)34Dc^4$. *In(2L)b79h1A* is named $tam^{10}$, as it behaves like a hypomorphic *tam* allele, exhibiting low viability, reduced compound eyes, and missing head and thoracic bristles when heterozygous with other alleles of *tam*.

(*BG:DS00941.5*) and mitochondrial DNA polymerase (*BG:DS00941.6*) genes were the only open reading frames between *Sos* and *Sop2*, we hypothesized that *tam* was the more proximal of these, *DNApol-γ125*. To test this hypothesis we sequenced this gene from the genomic DNA of all available mutant alleles ($tam^2$, $tam^3$, $tam^4$, and $tam^9$).

In the $tam^9$ allele a single nucleotide change (A → C) was found in exon 1 that substitutes a glutamine for alanine (Figure 10). This change was not found in any of the wild-type strains sequenced thus far. A mutation in the polymerase domain X (Glu → Val) was found in $tam^2$ mutation. This glutamine residue has been reported to be essential for the *E. coli* DNA polymerase catalytic function (Polesky *et al.* 1990) and is conserved in Drosophila, Saccharomyces, and Xenopus (Lewis *et*

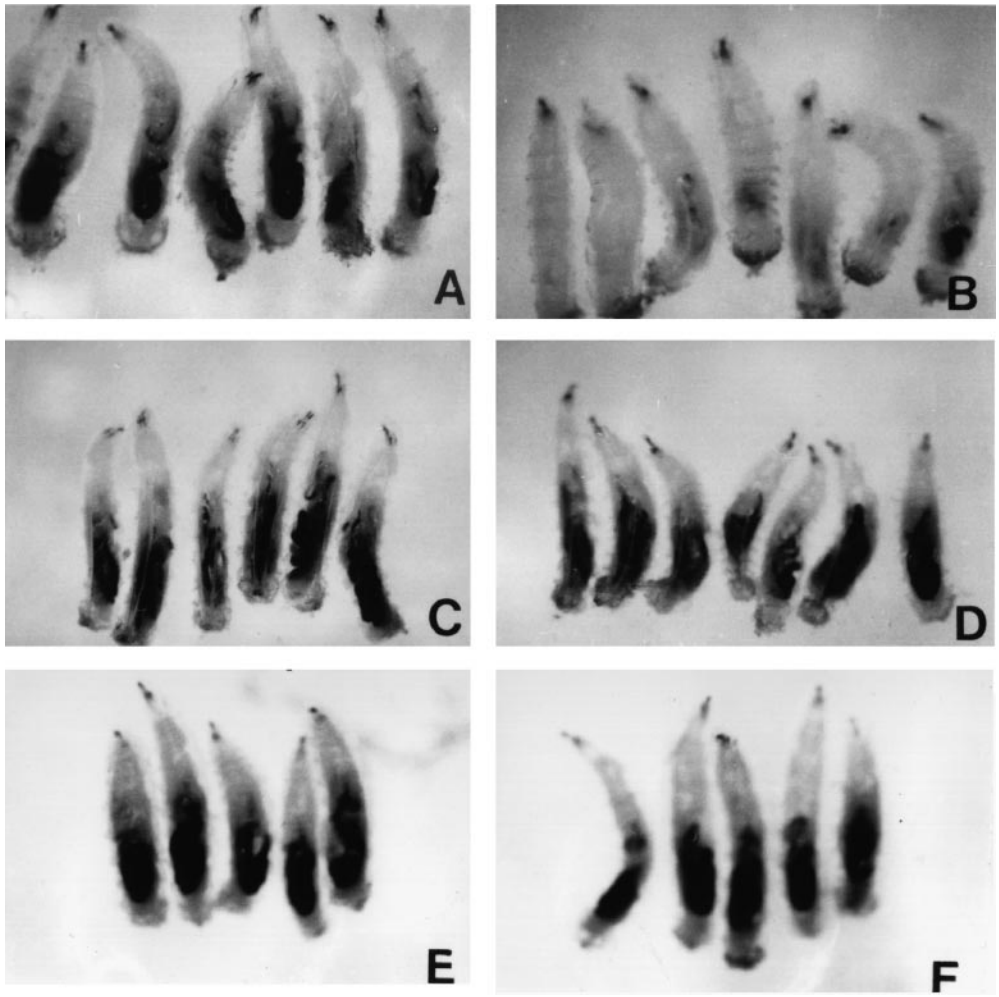



Figure 4.—Mutations in the *tam* gene resulted in prolonged foraging in third instar larvae. The larvae were grown in medium supplemented with bromophenol blue. Emptying of the guts as seen by the disappearance of blue coloration is a landmark of the transition from foraging to wandering stage. (A) Wild type (*P183*/*CyO-y*$^+$), 108 hr AEL. At this time larvae are at the wandering/foraging boundary. At this point in development wild-type larvae show blue coloration of the gut. (B) Wild type (*P183*/*CyO-y*$^+$), 117 hr AEL. Larvae at the wandering phase show reduced blue coloration in the gut. (C) *P183*/*P183* larvae at 108 hr AEL. (D) *P183*/$tam^3$ larvae at 108 hr AEL. (E) *P183*/*P183* larvae at 117 hr AEL. (F) *P183*/$tam^3$ larvae at 119 hr AEL. The retention of blue coloration at time points where the wild-type larvae show reduced coloration indicated that the mutant larvae did not wander on time.

**TABLE 4**

**Pattern of complementation of the recombined *tam*$^{9}$ is indistinguishable from a previously isolated allele (*tam*$^{3}$)**

| | *Df(2L)b80c1* | *In(2L)b82c44* | *In(2L)b83b22* | *In(2LR)b81a2* | *In(2L)b81l7* |
|---|---|---|---|---|---|
| *tam*$^{9}$ | −(0/289) | +(35/102) | −(0/105) | −(0/111) | +(45/146) |
| *tam*$^{3}$ | −(0/353) | +(40/101) | −(0/142) | −(0/164) | +(13/100) |

Minus indicates lethality and plus indicates viability. Numerator indicates the number of viable adult flies that were heterozygous for the chromosomal aberration and the *tam* alleles. The denominator indicates the number of flies with a marked balancer chromosome.

*al.* 1996). The mutation found in the *tam*$^{3}$ allele was a 5-bp deletion in exon 3 just outside the conserved DNA polymerase domain Z (Figure 10). The mutation identified in *tam*$^{4}$ was a single base pair deletion near the polymerase domain Z that results in a frameshift in the remainder of the coding region.

While the consequences of the change found in *tam*$^{9}$ for enzymatic activity are not known, the deletion found in *tam*$^{3}$ and *tam*$^{4}$ and the amino acid change found in *tam*$^{2}$ demonstrate that the catalytic subunit of the mitochondrial DNA polymerase is disrupted in *tam* mutants. Consistent with this notion we found that the pattern of distribution of mitochondria in the central nervous system as seen with the Mitotracker probe is

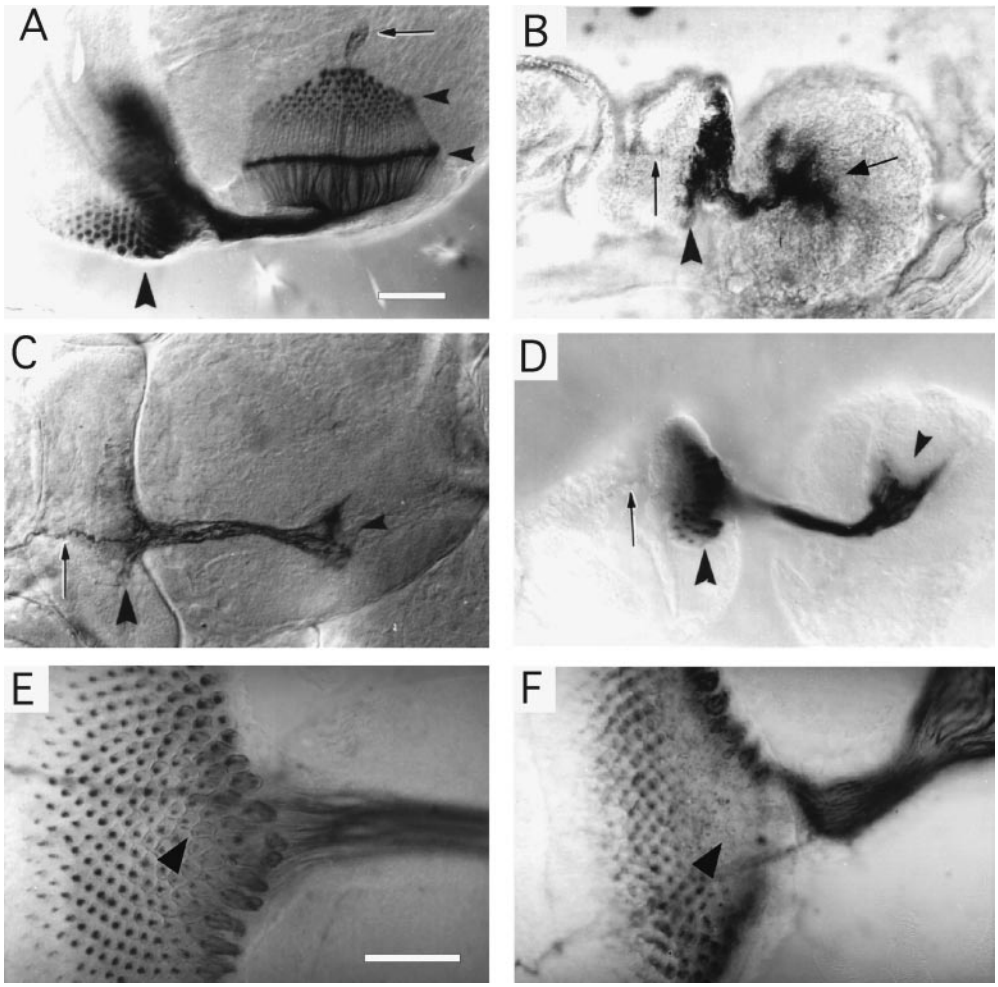



Figure 5.—Morphology of the developing adult visual system in *tam* mutants. The micrograph shows the developing adult visual system as seen by labeling with 24B10 primary antibody and HRP-conjugated secondary antibody. (A) Wild-type (Oregon-R) projection pattern viewed horizontally. In eye imaginal disc differentiating arrays of photoreceptors (large arrowhead) and the photoreceptor axonal projections of R1-R6 and R7-R8 into the optic lobe (small arrowheads) are seen. The larval optic nerve terminates deeper in the brain (arrow). (B) *P183/P183.* The photoreceptor differentiation is abnormal (arrowhead). Likewise photoreceptor axonal projection is disorganized (arrow). The larval optic nerve is present (thin arrow). (C) *P183/Df(2L)b80c1.* The defects in photoreceptor differentiation are apparent (large arrowhead). Abnormal projection pattern of photoreceptors in the optic lobe can also be seen (small arrowhead). The larval optic nerve is present (arrow). (D) *P183/tam*$^{3}$. As well as showing delayed photoreceptor differentiation, the eye disc is smaller and the area of differentiated photoreceptors is abnormal (large arrowhead). The projection pattern is also abnormal (small arrowhead). (E) Wild type. The older photoreceptor clusters located more posteriorly retain the clustered staining pattern as the differentiation proceeds more anteriorly. (F) *tam*$^{9}$/*tam*$^{9}$. The differentiating eye disc of the mutant larvae. In mutant eye discs a similar clustered staining pattern at the posterior regions of the eye disc appears not to be maintained. The scale bar in A represents 50 μm and is valid for B, C, and D. The scale bar in E represents 50 μm and is valid for F.

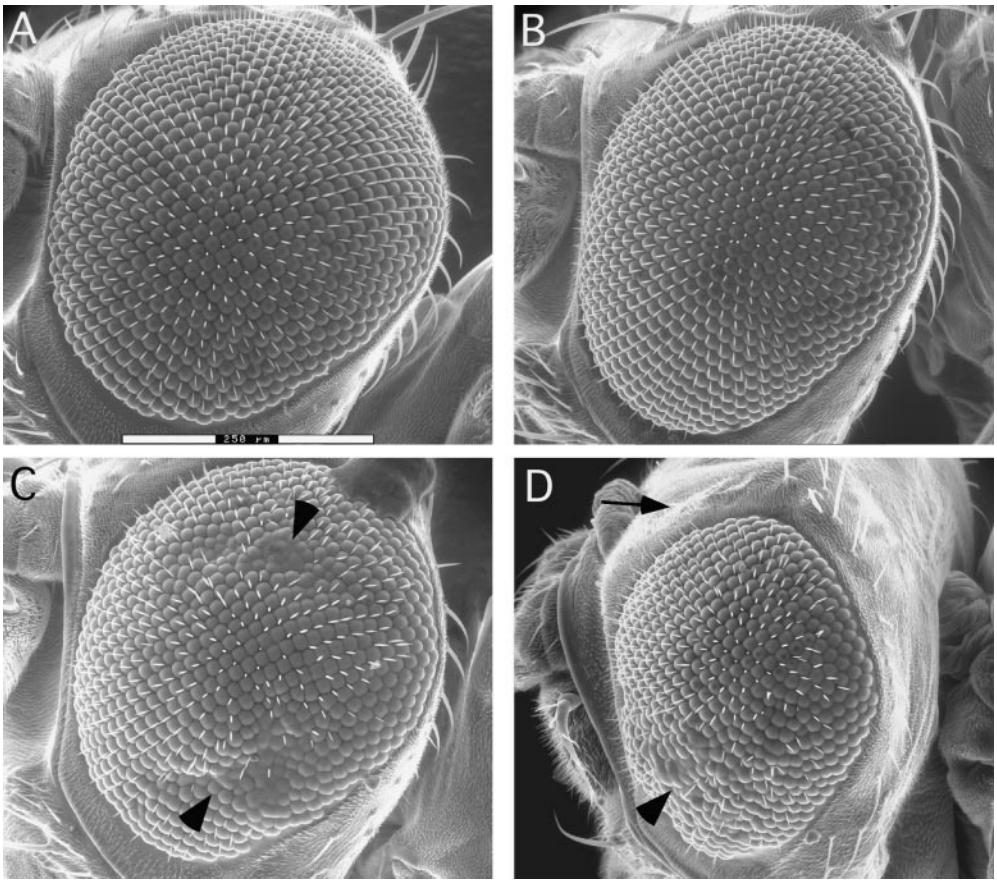



Figure 6.—ESEM scan of compound eye defects in *tam* escapers. (A) Wild-type control *In(2L)b79h1A*/*CyO-y*$^{+}$. (B) *In (2L)b79h1A/kuz*$^{3}$. *kuzbanian* (*kuz*) is the first known lethal complementation group to the left of *In(2L)b79h1A*; it is not disrupted by this inversion as seen by the wild-type pattern of the compound eye. (C) *In(2L) b79h1A*/*tam*$^{9}$. The compound eye is characterized by disorganized surface, fused ommatidia, and missing bristles (arrowheads). (D) *In(2L)b79h1A*/ *tam*$^{3}$. The arrowhead shows the rough surface, missing bristles, and fused ommatidia. These flies show a more severe disorganization of the eye than *In(2-L)b79h1A*/*tam*$^{9}$. The scale bar in A represents 250 μm and is valid for all panels.

severely disrupted by *tam* mutations (Figure 11). We concluded that the *tam* gene codes for the Drosophila DNA polymerase catalytic subunit (*DNApol-γ125*).

## DISCUSSION

To identify genes required for the larval response to light we screened a collection of pupal lethal lines using a population assay employed previously for the characterization of the wild-type response (Sawin-McCormack *et al.* 1995). The decision to use mutations that caused lethality at a later stage of development is supported by the observation that in Drosophila most of the genes required for visual system development are also required elsewhere for the viability of the organism (Thaker and Kankel 1992; reviewed by Miklos and Rubin 1996). This generalization was confirmed by Bier *et al.* (1989) who reported that the expression of most genes analyzed in enhancer trap strains was not restricted to one developmental stage. Investigations into the requirements of lethal mutations at different points in development by mosaic analysis yielded similar conclusions (reviewed by Miklos and Rubin 1996).

The sequencing of the mitochondrial DNA polymerase gene in all available *tam* alleles confirms the hypothesis, drawn from the genetics and published BDGP sequence data, that the *tam* gene codes for the catalytic subunit of the mitochondrial DNA polymerase. This is the first report in metazoans of a mutation in the mitochondrial DNA polymerase catalytic subunit (*DNApol-γ125*). Mutations in *tam* cause noticeable defects in the development of the adult visual system. Homozygous *tam* mutants also failed to undergo the behavioral changes characteristic of the wandering stage and remained in the food as foraging third instar larvae for a prolonged period. A more detailed analysis of the larval response to light in individual assays revealed that the lack of response to light of *tam* mutant larvae is due to a defect in locomotion. This motor deficit may also contribute to this mutant's failure to undergo the behavioral changes characteristic of the wandering stage.

Homozygous *tam* mutants die as a late third instar larvae. This observation suggests that the perdurance of the maternal contribution of *tam* gene product as well as of maternal mitochondria are sufficient to overcome the zygotic deficit of *tam* gene function well into late larval development. The locomotory phenotype of *tam*$^{3}$ and *tam*$^{9}$ mutations does not apparently represent the phenotype of complete absence of gene function as it is considerably worse when *in trans* with a deficiency that deletes the *tam* gene. In fact the molecular lesions found in these alleles support the notion suggested by the genetic analysis that the *tam*$^{3}$ and *tam*$^{9}$ mutations are hypomorphs. The *tam*$^{2}$ mutation is at present the

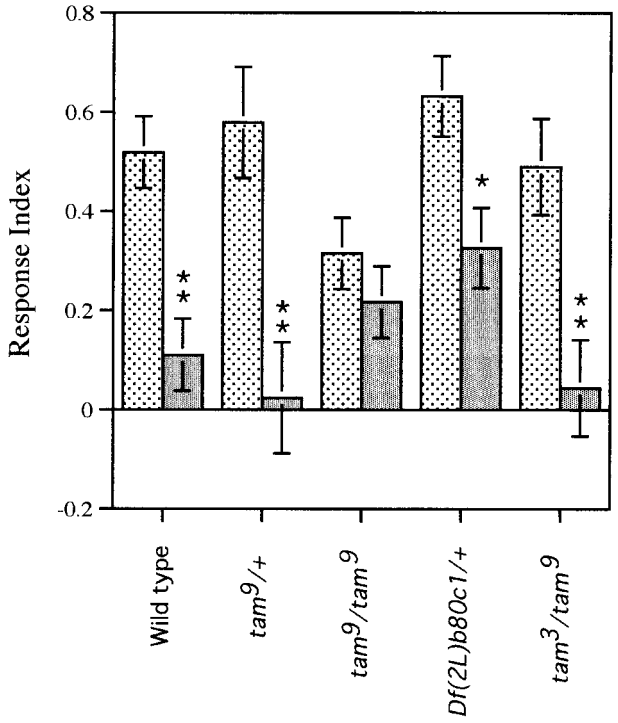



Figure 7.—Genetic analysis of photobehavior defect in *tam* mutants. In the Checker assay each genotype was assayed with lights on (test assay) and lights off (control assay). A response to light is present when the response index obtained with lights on (dotted bar) is significantly different from the response index obtained with lights off (shaded bar). The response index with lights on is not significantly different from that obtained with lights off in the homozygous *P183* (double mutant) and $tam^9$ (recombined) strains at any level of comparison, indicating a lack of response to light. In all other strains tested the response index obtained with lights on is significantly higher than that obtained with the lights off (**$P <$ 0.001, *$P < 0.01$ by Student's *t*-test). Wild-type control, lights on $n = 17$, lights off $n = 19$; $tam^9/+$, lights on $n = 17$, lights off $n = 16$; $tam^9/tam^9$, lights on $n = 17$, lights off $n = 15$; *Df(2L)b80c1*/+, lights on $n = 12$, lights off $n = 13$; $tam^9/tam^3$, lights on $n = 22$, lights off $n = 16$.

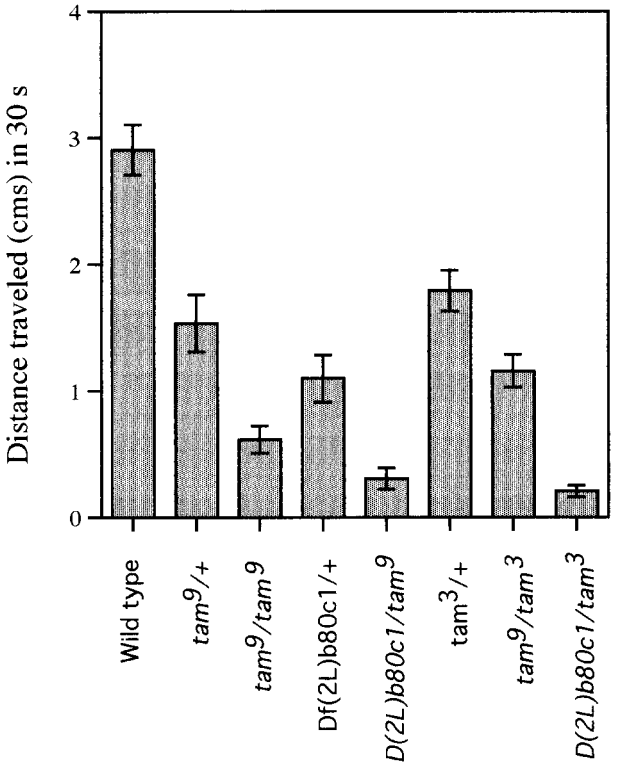



Figure 8.—Genetic analysis of locomotory defect in *tam* mutants. The graph depicts the mean distance traveled (SEM) in 30 sec on a nonnutritive substrate in the absence of light. The following comparisons were made using Student's *t*-test: $tam^9/tam^9$ *vs.* $tam^9/$*Df(2L)b80c1*, $T = 2.23$, $P = 0.033$; $tam^9/+$ *vs.* *Df(2L)b80c1*/+, $T = -1.71$, $P = 0.095$; *Df(2L)b80c1*/$tam^9$ *vs.* *Df(2L)b80c1*/+, $T = 3.89$, $P = 0.0006$; $tam^9/tam^9$ *vs.* $tam^9/tam^3$, $T = -3.10$, $P = 0.004$; $tam^3/+$ *vs.* $tam^9/tam^3$, $T = -2.97$, $P = 0.0052$; $tam^9/+$ *vs.* $tam^9/tam^3$, $T = 1.69$, $P = 0.099$; $tam^3/+$ *vs.* $tam^9/+$, $T = -1.08$, $P = 0.28$; *Df(2L)b80c1*/+ *vs.* *Df(2L)b80c1*/$tam^3$, $T = 4.67$, $P = 0.0001$. Wild-type control, $n = 19$; $tam^9/+$, $n = 22$; $tam^9/tam^9$, $n = 17$; *Df(2L)b80c1*/+, $n = 20$; *Df(2L)b80c1*/$tam^9$, $n = 18$; $tam^9/+$, $n = 22$; $tam^3/tam^9$, $n = 19$; *Df(2L)b80c1*/$tam^3$, $n = 19$.

best candidate for a null mutation. It presents a substitution in a glutamine residue of the DNA polymerase domain X essential for enzyme activity (Polesky *et al.* 1990).

Three other genes encoding for mitochondrial proteins with at least one allele affecting behavior have been identified in *D. melanogaster*. One is the *sluggish-A* (*slgA*) gene (Hayward *et al.* 1993), which encodes proline oxidase, a mitochondrial enzyme required for glutamate synthesis. *slgA* mutants display a locomotory and phototactic deficit similar to that described for *tam* mutants. The other two genes belong to the class of "bang or stress sensitive" mutants. They are *technical knockout* (*tko*) and *stress sensitive B* (*sesB*) and they encode a mitochondrial ribosomal protein and the adenine nucleotide translocase of the inner mitochondrial membrane, respectively (Royden *et al.* 1987; Zhang *et al.* 1998). Mutations in these loci cause flies to become temporarily paralyzed in response to physical jolt or stress. Similarly to *tam* mutants, *tko* mutants are weak and uncoordinated and grow significantly more slowly than wild type. Thus the Drosophila mutations isolated so far that affect mitochondrial function display a variety of nervous system-related phenotypes, some of which are similar to those described for *tam* mutants. These mutant phenotypes probably reflect the particular metabolic requirement of the nervous system.

The Drosophila mitochondrial DNA polymerase (pol-γ) is a heterodimer of two subunits (125 and 35 kD). This heterodimer contains two enzyme activities, a 5′→3′ DNA polymerase and a 3′→5′ exonuclease and is the sole enzyme responsible for mtDNA synthesis (Lewis *et al.* 1996). *In vitro* studies suggest that the 3′→5′ exonuclease activity proofreads errors during DNA synthesis and thus may play a role in the maintenance of the mitochondrial genetic integrity (Lewis *et al.* 1996). The cloning and sequence analysis of the Drosophila 125-

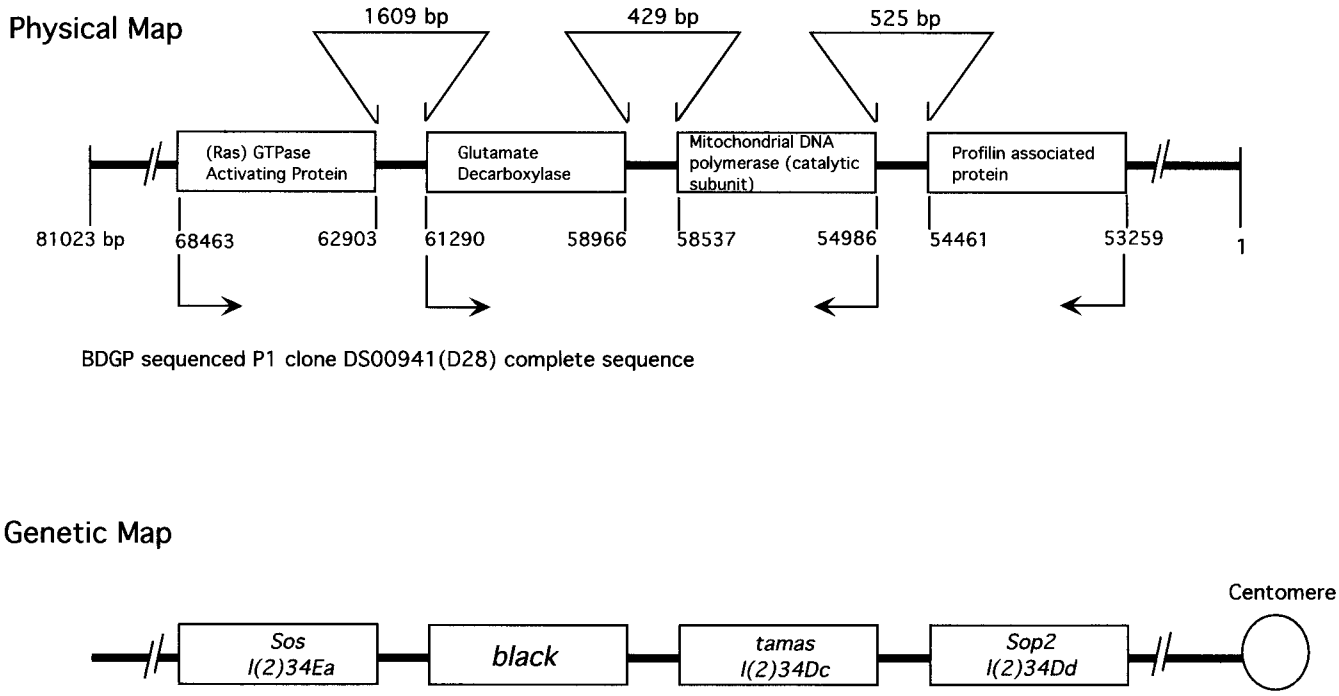



Figure 9.—Physical map of the vicinity of the *tam* locus. The map shows the nucleotide numbers of BDGP sequenced P1 clone DS00941, which starts at 1 and ends at 81023. Rectangular boxes indicate the transcriptional units. The direction of transcription is indicated by the arrows below the boxes. The distance between transcriptional units is indicated above and between the rectangular boxes. To aid comparison a genetic map is placed below the physical map. GenBank submitted sequences of guanine nucleotide exchange factor (*Sos*; GenBank accession no. L13173), *glutamate decarboxylase (black*; GenBank accession no. U01239), *mitochondrial DNA polymerase* (GenBank accession no. U60298), and profilin associated protein (*Sop2*; GenBank accession no. Y08999) were used to align the translational start and stop signal on the P1 DS00941 published sequence.

kD subunit demonstrated that this polypeptide contains both enzyme activities (Lewis *et al.* 1996). The gene for the Drosophila 35-kD subunit has also been cloned and sequenced (Wang *et al.* 1997). Interestingly, its genetic location is only 3.8 kb proximal to that of the catalytic subunit gene. It has been suggested that the β-subunit is required for the enzymatic efficiency and structural integrity of the holoenzyme (Lewis *et al.* 1996).

Mitochondrial diseases are a group of disorders caused by mitochondrial dysfunction. Most often the brain and/or muscle are affected, reflecting the energy requirement of these tissues. Several mtDNA mutations have been associated with specific clinical disorders (Moraes 1996). A smaller number of encephalomyopathies have been described that are likely due to mitochondrial DNA depletion caused by nuclear DNA mutations (Moraes *et al.* 1991; Ricci *et al.* 1992; Shanske 1992; Moraes 1996). Of those the most interesting is a fatal mitochondrial disease of infancy characterized by up to 95% mtDNA depletion in affected tissues. The pedigree of affected families suggested an autosomal recessive mode of inheritance (Mariotti *et al.* 1995). Two candidate genes tested in these studies were the mitochondrial single-stranded DNA-binding protein (*mtSSB*) required for strand displacement during mtDNA replication and the mitochondrial transcription factor A (*mtTFA*). Previous studies have indicated that *mtTFA* also plays a role in DNA replication as initiation of DNA synthesis requires an RNA primer (Parisi and Clayton 1991). The sequencing of *mtSSB* and *mtTFA* cDNAs isolated from probands showing mtDNA depletion did not show any difference from nonaffected individuals. It is possible that this early onset encephalomyopathy characterized by tissue-specific mtDNA depletion is caused by mutations that disrupt mtDNA replication and/or maintenance.

Mice lacking *mtTFA* die prior to embryonic day 10.5. The mutant embryos display growth retardation and delayed neural and cardiac development (Larsson *et al.* 1998). Given the requirement for an RNA primer for mtDNA replication, the phenotype of *mtTFA* homozygous knockout mice is likely to be very similar to animals lacking mtDNA replication. In fact homozygous knockout mice exhibit severe mtDNA depletion while heterozygous animals show tissue-specific mtDNA depletion. As expected from the studies of humans showing mtDNA depletion, the affected tissues in these mutant mice displayed a large number of abnormal mitochondria (Larsson *et al.* 1998).

Some aspects of the behavioral and morphological phenotype of *tam* mutant larvae are similar to that of *mtTFA* knockout mice and of humans showing an early onset encephalomyopathy associated with mtDNA depletion (Mariotti *et al.* 1995; Larsson *et al.* 1998).

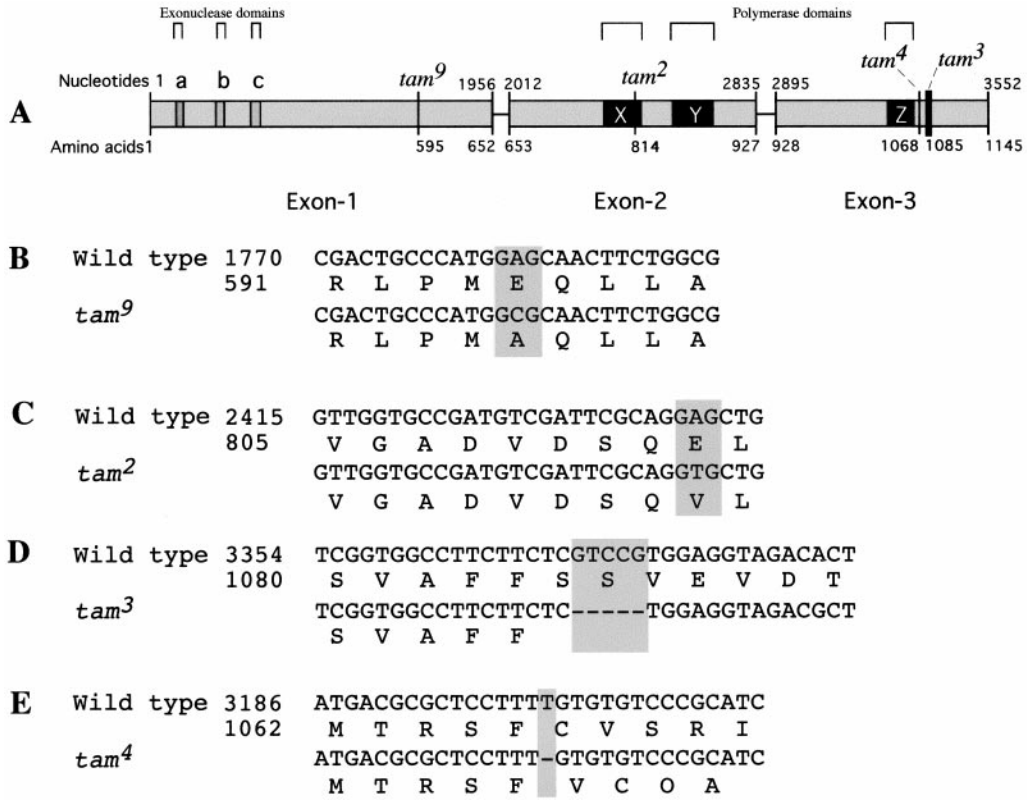



Figure 10.—Molecular lesions in *tam* mutant alleles. The genetic analysis and the BDGP sequencing project of the 34D region suggested that *DNApol-γ125* gene may represent the *tam* gene product. The sequence of the DNA polymerase catalytic subunit gene of all available *tam* mutant alleles was aligned to the respective sequence of the parental strains using Clustal X (see Table 1 for genotypes). The parental strain of the *tam*$^{9}$ allele was not available, hence it was aligned to sequence from an Oregon-R wild-type strain. (A) Schematic depiction of the structure of DNA*pol-γ125* gene (Lewis *et al.* 1996). Boxes a, b, and c represent the conserved 3′→5′ exonuclease domains, and X, Y, and Z represent the conserved DNA polymerase domains. Numbers above the exon boxes indicate the nucleotides from 1 to 3552 and numbers below the exon boxes indicate amino acids from 1 to 1145. (B) Sequence alignment of *tam*$^{9}$ allele against the Oregon-R wild-type sequence. The shaded box shows the mutation at nucleotide 1783 in *tam*$^{9}$ (A→C); this causes an amino acid change (glutamine to alanine). (C) Sequence alignment of *tam*$^{2}$ allele against the corresponding parental sequence showing a 5-bp deletion at position 3370. (D) Sequence alignment of *tam*$^{3}$ against the corresponding parental strain. The alignment shows an amino acid change from glutamine to valine at position 813 in a highly conserved polymerase domain of the protein. (E) Shows the alignment of *tam*$^{4}$ against the corresponding parental sequence. The alignment resulted in identification of a single base pair deletion at position 3201; this causes a frameshift of the remaining coding region.

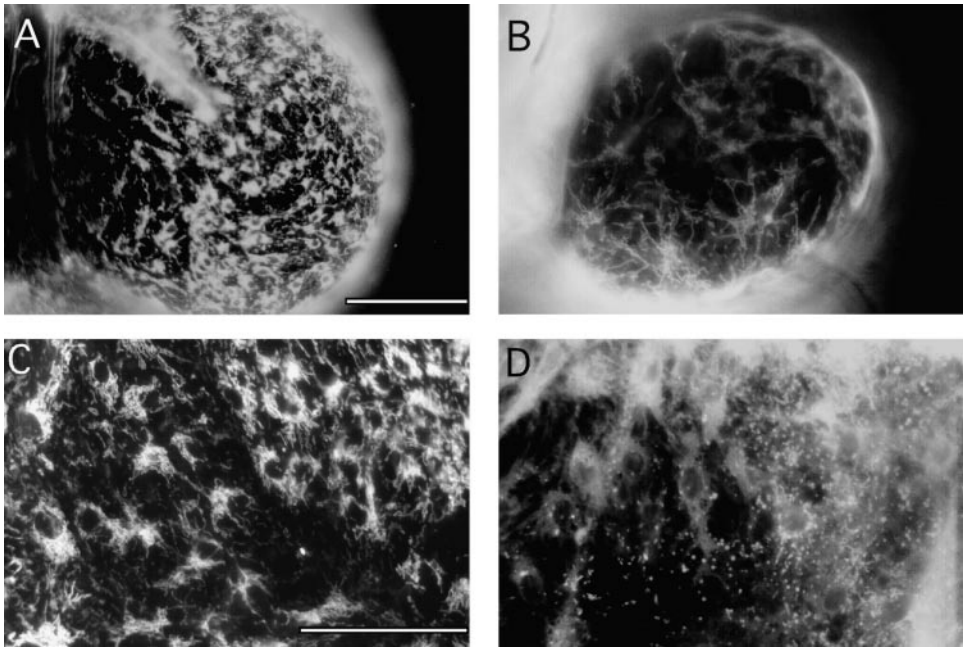



Figure 11.—Visualization of mitochondrial mass using Mitotracker probe. Third instar brain hemispheres stained with Mitotracker probe. (A) Wild-type brain hemisphere, 96 hr AEL. (B) *tam*$^{9}$/*tam*$^{9}$, 96 hr AEL. (C and D) Higher magnification of wild-type and mutant brain hemispheres, respectively. Scale bar in A represents 50 μm and is valid for B. Scale bar in E represents 50 μm and is valid for D.

*tam* mutant larvae and *mtTFA* mutant mice both display growth retardation and delayed neural development. The infant proband in this particular pedigree displayed severe motor delay during the first month of life and subsequent arrest of psychomotor development concomitant to mild global atrophy of the brain (Mariotti *et al.* 1995).

The control of mtDNA maintenance is an important issue not only in inherited encephalomyopathies but also in the treatment of AIDS. The mitochondrial myopathy seen in AIDS patients after prolonged exposure to AZT is due to the incorporation of this drug in mtDNA by mtDNA polymerase causing inhibition of mtDNA replication (Dalakas *et al.* 1990; Arnaudo *et al.* 1991; Ricci *et al.* 1992). Drosophila larvae mutant for the *tam* gene may provide a useful model system to study control of mtDNA replication. It provides the unique opportunity to screen for second-site mutations that modify the *tam* phenotype and hence allow us to identify genes that may be involved in the same pathway.

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