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# *technical knockout*, a Drosophila Model of Mitochondrial Deafness

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ABSTRACT

Mutations in mtDNA-encoded components of the mitochondrial translational apparatus are associated with diverse pathological states in humans, notably sensorineural deafness. To develop animal models of such disorders, we have manipulated the nuclear gene for mitochondrial ribosomal protein S12 in Drosophila (*technical knockout, tko*). The prototypic mutant *tko*$^{25t}$ exhibits developmental delay, bang sensitivity, impaired male courtship, and defective response to sound. On the basis of a transgenic reversion test, these phenotypes are attributable to a single substitution (L85H) at a conserved residue of the tko protein. The mutant is hypersensitive to doxycyclin, an antibiotic that selectively inhibits mitochondrial protein synthesis, and mutant larvae have greatly diminished activities of mitochondrial redox enzymes and decreased levels of mitochondrial small-subunit rRNA. A second mutation in the *tko* gene, Q116K, which is predicted to impair the accuracy of mitochondrial translation, results in the completely different phenotype of recessive female sterility, based on three independent transgenic insertions. We infer that the *tko*$^{25t}$ mutant provides a model of mitochondrial hearing impairment resulting from a quantitative deficiency of mitochondrial translational capacity.

MITOCHONDRIAL DNA (mtDNA) in metazoans encodes a small subset of the polypeptide subunits of the mitochondrial redox complexes, plus the RNA components of the mitochondrial translational apparatus (Wolstenholme 1992). In humans, mtDNA mutations are associated with a wide range of pathological states, typically manifesting as multisystem (syndromic) disorders affecting tissues highly dependent on mitochondrial energy, such as brain, muscle, heart, and the sensorineural epithelia (Fadic and Johns 1996). Each mutation is associated with a distinct spectrum of clinical phenotypes, but sensorineural deafness is a common feature, notably in the case of mutations affecting mitochondrial rRNA and tRNA genes (reviewed in Jacobs 1997; Fischel-Ghodsian 1999). Indeed, deafness is the sole pathological symptom in many individuals harboring mitochondrial tRNA mutations.

Although some progress has been made in understanding how mtDNA mutations lead to impaired bioenergetic function at the cellular level, many issues remain unresolved. For example, it is unclear whether mtDNA mutations in components of the translational apparatus produce disease by quantitative or qualitative interference with mitochondrial translation. The tissue specificity of mitochondrial disease remains largely unexplained, and the pathological process in the whole organism is little understood. Moreover, research in this area is hampered by the lack of animal models resulting from the intractability of manipulating mtDNA *in vitro*.

To circumvent these difficulties, we are exploiting a previously isolated mutant in the fruit fly Drosophila that has phenotypic features strongly reminiscent of mitochondrial disease. This mutant (*technical knockout*, *tko*, mutant allele *tko*$^{25t}$) has a phenotype of bang sensitivity (Judd *et al.* 1972; Shannon *et al.* 1972; Ganetzky and Wu 1982), *i.e.*, temporary paralysis resulting from mechanical vibration, associated with a failure of signaling from mechanoreceptor neurons (Engel and Wu 1994). Such a phenotype represents a potential parallel with forms of sensorineural deafness in humans that result from a failure of signaling from the mechanosensory receptor cells of the inner ear.

The *tko* mutant phenotype can be fully complemented transgenically by a 3.2-kb fragment of genomic DNA containing a single transcription unit that encodes a homologue of bacterial ribosomal protein S12 (Royden *et al.* 1987). The coding sequence has two features indicating a role for this protein in mitochondria (Shah *et al.* 1997). First, phylogenetic analysis places it much closer to the S12 proteins of eubacteria and other organelles than to the identified cytosolic ribosomal homo-

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logue found in yeast. Second, in comparison with its eubacterial and organellar homologues, it has an N-terminal extension with features typical of a mitochondrial targeting presequence. A similar N-terminal extension is found in other metazoan homologues (Shah *et al.* 1997), and reporter assays in human cells have confirmed that the human homologue MRPS12 is indeed a mitochondrially localized gene product (Mariottini *et al.* 1999).

Thus far, $tko^{25t}$ is the only viable mutant allele of the gene that has been isolated, all others being recessive larval lethals. $tko^{25t}$ is itself recessive, and is also female hemizygous lethal, presumably because it is a hypomorph for which half the normal gene dosage is insufficient to complete development. Sequence analysis of the coding region of the mitochondrial ribosomal protein S12 encoded at this locus in wild-type (Canton-S) and $tko^{25t}$ flies revealed a single amino-acid difference (L85H) affecting a phylogenetically conserved leucine residue (Shah *et al.* 1997). The analogous substitution, L56H, introduced into the *Escherichia coli* homologue *rpsL*, functionally impairs assembly of the gene product into active ribosomes (Toivonen *et al.* 1999). However, formal proof that the L85H coding region mutation is the cause of the $tko^{25t}$ phenotype is lacking. Northern blotting of RNA from wild-type and $tko^{25t}$ mutant flies revealed no overall differences in expression level or transcript size (Shah *et al.* 1997). However, this did not rule out the possibility that the mutant allele carries a regulatory mutation outside of the coding region, with a more subtle, tissue-specific effect. Direct evidence that the mutation affects mitochondrial protein synthesis and in what way is also lacking.

In this article we report the results of genetic, biochemical, and behavioral experiments that indicate that the $tko^{25t}$ mutant represents a valid model for mitochondrial hearing impairment in humans and contribute important information regarding the molecular mechanism of this disorder. First, we reveal novel details of the $tko^{25t}$ mutant phenotype involving defects in developmental timing, courtship, and hearing. Second, we show by various approaches that the mutant phenotype is associated with a quantitative deficiency of mitochondrial translation. Third, we demonstrate, by a transgenic reversion test, that the L85H substitution is indeed the cause of the main features of the $tko^{25t}$ mutant phenotype.

## MATERIALS AND METHODS

**Flies and fly culture:** $tko^{25t}$ mutant flies were kindly supplied by Dr. C.-F. Wu. The $tko^{3}$ line $tko^{3}$/FM7a/Dp(1;2;Y)$w^{+}$ was obtained from the *Drosophila* Stock Center (Bloomington, IN) and outcrossed to an existing FM7 balancer strain. Other strains used were Canton-S (wild type), 0713 (inbred wild type), and $w^{1118}$ (for micro-injection). Flies were cultured on a standard oatmeal and molasses medium seeded with live yeast, to which was added various antibiotics (doxycyclin or streptomycin) as indicated in the text or figure legends. For behavioral and developmental analyses flies were maintained at 25° on a 12 hr:12 hr light:dark cycle, unless otherwise stated. To assess developmental timing neutral red (an inert dye) was added to the media of both wild-type and $tko^{25t}$ mutant flies.

**Behavioral assays:** Bang sensitivity was measured, essentially as in Royden *et al.* (1987), by vortexing individual adult flies at maximum speed for 10 or 15 sec (see figure legends) in a standard vial and recording the time taken for recovery from paralysis. Reactivity (locomotor activity in response to a mechanical disturbance) was measured for individual flies by tipping them onto a gridded open-field chamber (10 × 10 × 1 cm) through a closed entry port. After tapping the chamber lightly 10 times in succession, the fly's movement was recorded over a 10-min period (for further details see O'Dell and Burnet 1988). Reactivity was plotted as the number of centimeters traveled during each succeeding 1-min interval. Spontaneous activity was measured in a circadian rhythm machine (Konopka *et al.* 1994). Courtship analysis was carried out as described previously (O'Dell *et al.* 1989). Hearing was measured using a modified version of the assay used by Von Schilcher (1976) and Eberl *et al.* (1997). Males were collected in batches of 10 within 6 hr of eclosion and wings were removed surgically the next day. At age 5–7 days six males from each batch were transferred to a mating chamber and exposed to 10 min of species-specific courtship song (Ritchie *et al.* 1999) after 5 min of silence. For each batch, the song was played at one of six different volumes (60, 70, 80, 90, 100, or 110 dB). The number of males (maximum of 6) attempting to copulate with each other was scored during each of three 10-sec intervals—at 4 min 40 sec, 6 min, and 14 min 30 sec—from the start of the experiment (*i.e.*, before song, after 1 min of song, and after 10 min of song). In each case, to control for any differences in reactivity or spontaneous homosexual courtship, the effect of song at 1- and 10-min time points was calculated as the mean number of flies exhibiting courtship behavior after song minus the number before song.

**Oligonucleotides:** All oligonucleotides were purchased from DNA Technology (Aarhus, Denmark). Oligonucleotides used for PCR and PCR *in vitro* mutagenesis (see Figure 5 for their approximate locations) were as follows, with all sequences given in the 5′ to 3′ direction. For the *tko* coding strand

tko-53: CGGGATCCAGATAATCGTAGGACAGGTCGGCAGA,

H85L-1: GTGCTGGTGCGCCTCTCCACCGGCAAG,

Q116K-1: GTGGGGCGTCTGAAGGACGTGCCCGG,

tko-32: ACCACTTCCTAATCACGTTGTTCAAATG,

tko-54: CGGGATCCAGATAATCGTAGGACAGGT, and

tko-56: CGGGATCCGAATATTAGCTTTAAGGCACCCG.

For the noncoding strand

tko-52: CGGAGTTTTGTTCAGTAGTTTTCTGTTC,

H85L-2: TTGCCGGTGGAGAGGCGCACCAGCAC,

Q116K-2: CCGGGCACGTCCTTCAGACGCCCCAC,

tko-33: CGGGATCCTGATATTATATAGCTTGCAATCAG AGATA,

tko-34: CGGGATCCTGATATTATATAGCTTGCAAT, and

tko-36: CGGAATTCTGATATTATATAGCTTGCAAT.

For the mutagenic primers H85L-1, H85L-2, Q116K-1, and Q116K-2 the nucleotides that introduce the mutation are underlined. Restriction sites used for cloning (in every case *Bam*HI, except *Eco*RI for tko-36) are double underlined. Note

that primers tko-54 and tko-34 overlap, respectively, tko-53 and tko-33, but are slightly shorter, giving better compatibility with the mutagenic primers. For DNA sequencing of the cloned 3.2-kb *Bam*HI fragment containing the *tko* gene, standard vector primers plus the following were used, in addition to some of those listed above: *P*-in, GATGAAATAACATAAG GTGGTCCCGTCG (*P*-element-specific); tko-55, CTAAATAG CTTCCGAGCAAG [coding strand, 5′ untranslated region (UTR)]; tko-51, GAACAGAAAACTACTGAACAAAACTCCG (coding strand, 5′ UTR just upstream of the translation start); tko-35, GCAGCTAATGGCCACCAAATC (noncoding strand, near start of intron); tko-I2, GGTCGCAGTTTAGGAGACAA TAGGTG (coding strand, near middle of intron); and tko-31, CATTTGAACAACGTGATTAGGAAGTGGT (noncoding strand, end of coding sequence). For PCR amplification of fragments of Drosophila mtDNA to create probes for Northern hybridization, the following primers (supplied by Genset, Paris), were used. For 16S rRNA,

D16S-1: AGAAACCAACCTGGCTTACACCGG
D16S-2: AAGACATGTTTTTGTTAAACAGGCGAATA.

For 12S rRNA,

D12S-1: TCATTCTAGATACACTTTCCAGTACATC
D12S-2: ACCGCGACTGCTGGCACCAATTTAGT.

**PCR cloning and *in vitro* mutagenesis:** Except where stated, all steps were carried out using standard procedures (Sambrook *et al.*1989). The 3.2-kb *Bam*HI fragment containing the *tko* gene was cloned from *tko*$^{25t}$ genomic DNA by a two-step PCR strategy illustrated in Figure 5. Genomic DNA was digested with *Bam*HI (manufacturer's buffer; New England Biolabs, Beverly, MA), circularized by ligation at low DNA concentration, then amplified by PCR using primers tko-52 and tko-32 in a reaction containing 20 pmol of each primer plus 0.6 units of DynaZyme II DNA polymerase (Finnzymes, Espoo, Finland), with 30 cycles of annealing at 57° for 1 min and extension at 72° for 2 min in the manufacturer's recommended buffer containing 1.5 mm $MgCl_2$. The 1.7-kb product was cloned using the TA cloning kit (Invitrogen, Groningen, Netherlands), and its identity was verified as deriving from the *tko* gene by DNA sequencing, using vector primers. The sequence around the artificially ligated *Bam*HI site of this clone (gtkoII) was determined and was used to design oligonucleotide primers tko-53 and tko-33, which were then used to amplify the entire 3.2-kb fragment using the Expand high fidelity PCR system (Boehringer Mannheim, Mannheim, Germany) in the manufacturer's buffer over 30 cycles of annealing for 1 min at 60°, followed by extension for 7 min. The final product was digested with *Bam*HI and cloned into the *Bam*HI site of the *P*-element vector pP{CaSpeR-4} (Thummel and Pirrotta 1992). For PCR *in vitro* mutagenesis, this construct, pP{CaSpeR-4}/tko(25t)-*Bam*HI, was used as template DNA in a two-step procedure (see scheme outlined in Figure 5). In the first step, tko-54 plus mutagenic primer H85L-2 and tko-34 plus mutagenic primer H85L-1 were used in separate reactions. High-fidelity PCR products created using the mutagenic primers were purified on QIAquick spin columns, diluted 100-fold, mixed together, and used as template in a second-round PCR reaction to generate a full-length product with a terminal *Bam*HI restriction site for recloning in pP{CaSpeR-4}. To introduce the Q116K substitution, an equivalent procedure was employed, starting from the *in vitro*-reverted construct using mutagenic primers Q116K-1 and Q116K-2. The 5′ primer tko-56 was used instead of tko-54, since it worked more efficiently in combination with Q116K-2. The 3′ primer tko-36, in which the terminal *Bam*HI site is replaced by *Eco*RI, was substituted for tko-34. Colonies containing appropriate recombinant plasmids were identified by single-colony PCR using *tko*-specific primers, and plasmid DNA was then isolated, using appropriate Promega Wizard kits for DNA sequencing and eventual micro-injection.

**DNA sequencing and informatics:** Plasmid DNAs and PCR products were sequenced using dye-terminator chemistry on the ABI 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA), with kit reagents supplied by the manufacturer, plus standard (M13R, T7) and customized primer oligonucleotides as listed above. DNA sequences were analyzed using the GCG package (Program Manual for the Wisconsin Package, 1994).

**Mitochondrial preparation:** Mitochondria were isolated by differential centrifugation of third instar larvae ground in buffer containing 0.22 m sucrose, 0.12 m mannitol, 10 mm Tricine, pH 7.6, and 1 mm EDTA, as described previously (Alziari *et al.* 1981). Protein determination was performed (Bradford 1976) using the Bradford reagent (Bio-Rad, Richmond, CA). For enzymatic assays, mitochondria ($\sim 1$ mg ml$^{-1}$) were sonicated for 6 sec at 4°, frozen, and thawed, except when used for ATP synthesis determination.

**Assays of mitochondrial redox activities:** All activities except ATP synthesis were measured at 28° and expressed in nanomoles per minute per milligram. Complex I (NADH:ubiquinone oxidoreductase) activity was monitored by NADH oxidation at 340 nm ($\varepsilon$ = 6220 m$^{-1}$ cm$^{-1}$) in pH 7.2 buffer containing 35 mm $NaH_2PO_4$, 5 mm $MgCl_2$, and 2.5 mg/ml bovine serum albumin (BSA), in the presence of 2 μg/ml antimycin, 2 mm potassium cyanide, 97.5 μm ubiquinone, 0.13 mm NADH, and 40 μg mitochondrial protein (Hatefi 1978a). Only the rotenone-sensitive activity was recorded. For measurements of complex III (ubiquinol:cytochrome *c* oxidoreductase), ubiquinol was obtained by reduction of ubiquinone with dithionite and then extraction with cyclohexane. After evaporation of the cyclohexane, ubiquinol was dissolved in ethanol and stabilized with 10 mm HCl. Cytochrome *c* reduction was monitored at 550 nm ($\varepsilon$ = 18,500 m$^{-1}$ cm$^{-1}$) in pH 7.2 buffer containing 35 mm $NaH_2PO_4$, 5 mm $MgCl_2$, and 2.5 mg/ml BSA in the presence of 2 mm KCN, 2 μg/ml rotenone, 15 μm ubiquinol, 15 μm cytochrome *c*, and 5 μg mitochondrial protein (Hatefi 1978b). The nonenzymatic reduction of cytochrome *c* with ubiquinol (in the absence of mitochondria) was subtracted from this measurement. Complex IV (cytochrome *c* oxidase) activity was determined by oxidation of partially reduced cytochrome *c* (reduced OD − oxidized OD = $\sim 0.75$), monitored at 550 nm ($\varepsilon$ = 18,500 m$^{-1}$ cm$^{-1}$) in pH 7.4 buffer containing 30 mm $KH_2PO_4$, 1 mm EDTA, 56 μm cytochrome *c*, and 5 μg mitochondrial protein (Errede *et al.* 1978). Citrate synthetase activity was assayed by the appearance of thionitrobenzoic acid (yellow) derived from the reduction of dithio-*bis*-nitrobenzoic acid (DTNB-colorless) with coenzyme A, monitored at 412 nm ($\varepsilon$ = 13,600 M$^{-1}$ cm$^{-1}$) in 100 mm Tris-HCl, pH 8 buffer, 2.5 mm EDTA, 37 μm acetyl-CoA, 75 μm DTNB, 300 μm oxaloacetate, and 5 μg mitochondrial protein (Shepherd and Garland 1969). Mitochondrial ATP synthesis was measured using a technique adapted from that described by Wibom *et al.* (1991), which is based on the luminescence of luciferin in the presence of luciferase, monitored with a BioOrbit 1251 luminometer, which is proportional to the ATP concentration in the test medium (Deluca 1969). Kinetics were recorded at 25°, pH 7.5, with 50 μl of the incubation medium containing 0.15 mm ADP, 54 μm APP, and 1 μg mitochondrial protein. After 2 min of incubation, 5 μl of substrate and 100 μl of reagent were added simultaneously and fluorescence was monitored for 1 min. The final concentrations of substrates were 10 mm glutamate + 10 mm malate, 20 mm α-glycerophosphate, or 3.3 mm ascorbate + 80 μm TMPD. Calibration was performed at the end of each measurement by the addition of 200 pmol of ATP.

***P*-element transgenesis:** DNA from pP{CaSpeR-4} constructs

was micro-injected into recipient $w^{1118}$ embryos together with DNA from *P*-element transposase-encoding plasmid pUChsΔ2-3 (Rio 1996), using standard methods (Spradling 1986). Injected flies surviving to adulthood were mated to virgin $w^{1118}$ mutants and their red-eyed progeny were selected. Strains derived from these putative transgenic flies were retained for behavioral and molecular characterization (see results).

**DNA extraction and Southern hybridization:** Aliquots of 20 flies were ground in 500 μl lysis buffer [100 mm Tris-HCl, pH 8.5, 80 mm NaCl, 5% (w/v) sucrose, 0.5% (w/v) SDS, 50 mm EDTA, pH 8.0], frozen at −20°, and then incubated at 70° for 30 min. KCl was added to 400 mm and samples were placed on ice for 30 min. The precipitates were removed by centrifugation and the supernatants were extracted twice with phenol/chloroform (1:1) and precipitated with 0.75 volume isopropanol. DNA precipitates were washed with 70% ethanol, dried, and dissolved in 45 μl TE (10 mm Tris-HCl, 1 mm EDTA, pH 8.0) containing 20 μg/ml boiled RNase A. One-third of each DNA aliquot was digested overnight with *Eco*RV (manufacturer's buffer and recommended conditions; New England Biolabs), and fractionated on a 1% agarose gel. Depurination, capillary blotting to MagnaCharge nylon membrane (Osmonics, Inc., Westborough, MA), and UV cross-linking used standard methods (Sambrook *et al.* 1989). The blot was prehybridized for 45 min at 65° in 250 mm phosphate buffer, pH 7.2, 7% SDS, and 1 mm EDTA and hybridized overnight at 65° in the same buffer to a probe covering the *tko*-coding region, which was derived by PCR amplification of the cloned segment of genomic DNA using primers tko-51 and tko-31. The probe was radiolabeled by random-primed DNA synthesis in the presence of [α-$^{32}$P]dCTP (3000 Ci/mmol; Amersham, Pharmacia UK, Buckinghamshire). The blot was washed at 65° for 20 min with 3× SSC, 0.1% SDS, twice for 20 min with 0.3× SSC, 0.1% SDS, and finally for 10 min in the same buffer before autoradiography.

**RNA extraction and Northern hybridization:** Third instar larvae (~100 mg) were homogenized in Trizol reagent (GIBCO-BRL, Gaithersburg, MD) and total RNA was extracted according to the manufacturer's instructions. Between 5 and 30 μg of RNA from larvae of each strain were ethanol precipitated, air dried, and resuspended in 4.5 μl pyrocarbonic acid diethyl ester-treated water, to which was added 2 μl 5× MOPS buffer (Sambrook *et al.* 1989), 3.5 μl formaldehyde, and 10 μl formamide. Samples were heated at 65° for 15 min, chilled on ice, and loaded onto a 1% agarose/formaldehyde gel (Sambrook *et al.* 1989) for electrophoresis. The gel was capillary blotted and hybridized as for Southern hybridization, using randomly labeled probes for Drosophila mitochondrial rRNAs (PCR products as indicated above) or cytosolic ribosomal protein rp49 mRNA (cloned genomic DNA). Filters were washed at 65°, once for 20 min with 3× SSC, 0.1% SDS and twice for 20 min with 0.3× SSC, 0.1% SDS before autoradiography and quantitation by densitometry.

## RESULTS

**The $tko^{25t}$ mutant phenotype:** Previous studies of $tko^{25t}$ mutant flies demonstrated bang sensitivity associated with mechanoreceptor failure and a possible structural abnormality of mechanosensory organs, as well as female hemizygous lethality. To document the phenotype more precisely in a developmental context, we studied inbred $tko^{25t}$ flies, as well as some that had been partially outbred for several generations by being maintained as heterozygotes with the FM7 balancer chromosome (Merriam 1969). As illustrated in Figure 1a, when tested 3 hr after eclosion for sensitivity to mechanical vibration by 10 sec of vortexing, $tko^{25t}$ males or homozygous females showed a strong bang-sensitive phenotype with a mean recovery time of >30 sec. The time to recovery was somewhat longer in $tko^{25t}$ flies that had been outbred for several generations. When retested 4 days later, the bang-sensitive phenotype had almost completely disappeared, with a mean time to recovery of <5 sec (data not shown). These findings suggest that the behavioral consequences of mechanoreceptor failure can be at least partially compensated both in the lifetime of an individual fly, *e.g.*, via an adaptation mechanism, and also to some extent in the course of evolution, via selection during inbreeding.

The adult fly, even when inbred, still suffers a clear sensory and/or behavioral defect, as demonstrated by the results of activity measurements (Figure 1b). The mean reactivity (measured as the distance traveled in the first minute in response to a mechanical disturbance) of the mutant flies was less than half that of the wild-type flies. However, mutant males and females showed normal activity in a circadian rhythm machine (data not shown) and were rhythmic, indicating that their activity defect is specifically in reactivity. Courtship analysis, in which wild-type and $tko^{25t}$ flies were mated in all combinations of gender and genotype (Table 1), also revealed evidence for sensory/behavioral abnormalities. The $tko^{25t}$ males were rejected by wild-type females, but wild-type males were equally successful at courting either $tko^{25t}$ or wild-type females. $tko^{25t}$ males were moderately successful in courting $tko^{25t}$ females, but also showed a substantially prolonged courtship and reduced mating time compared with wild type.

Flies were tested for a hearing deficit by exposure to experimentally generated courtship song batches of males whose wings had been surgically removed (Von Schilcher 1976; Eberl *et al.* 1997). Under such conditions, males that hear normally respond by courting each other. The test was internally corrected for differences in reactivity or spontaneous courtship behavior between mutant and wild-type flies. Wild-type males showed a clear response to courtship song at 70–90 dB, but $tko^{25t}$ males showed only a very slight response at 100 dB (Figure 2). We infer that the mutant has a severe hearing deficiency. All of these aspects of the mutant phenotype are consistent with a primary defect in mechanoreceptor function, as discussed below.

Mutant flies showed several other developmental abnormalities that are not obviously attributable to a sensorineural deficit. At 25° they took ~2–3 days longer to develop than their wild-type counterparts, eclosing on days 12–13 instead of on days 10–11 (Figure 3). The effect was more marked in males than in females. In three independent experiments, each involving the offspring from 30 females, mutant males showed a mean developmental delay to eclosion of 2.40 ± 0.10 days,

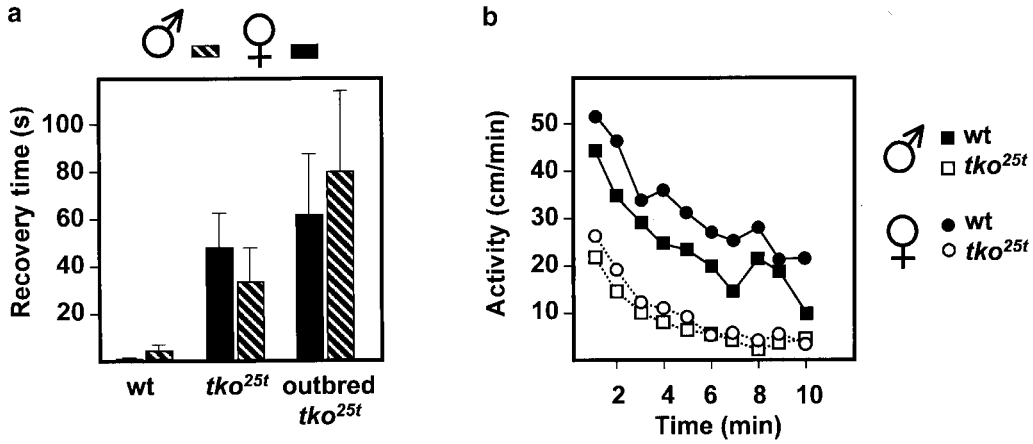


FIGURE 1.—Bang-sensitive phenotype of *tko*$^{25t}$ mutant flies. (a) Recovery times from paralysis after 10 sec of vortexing (mean ± SE of measurements on 10 individual flies of each sex and genotype) of wild-type and *tko*$^{25t}$ mutant flies, tested between 2–4 hr after eclosion, both with and without outbreeding. Note large standard errors for mutant flies due to "outliers" with very long recovery times. (b) Reactivity measurements on wild-type and *tko*$^{25t}$ adults (3 days old), plotted as mean distance traveled (centimeters traveled per minute) in the open-field chamber over the 10-min period after the initial mechanical disturbance. Means are from 10 flies of each sex and genotype. For clarity, standard errors are not shown on the figure, but were generally in the range of 1–4 cm for each data point plotted; hence, the differences between mutant and wild-type flies are highly significant.

whereas the delay in females was 2.17 ± 0.06 days. Daily observations of the developing flies indicated that the delay occurred almost exclusively during the larval stages, mainly the second and third larval instars. No delay was evident during embryogenesis; *i.e.*, mutant larvae hatched at the same time as wild type, whereas pupariation was at least 2 days late in the mutants. The mutant allele had no maternal effect on developmental timing. Heterozygous offspring of *tko*$^{25t}$ or wild-type mothers eclosed at the same time as each other and as wild-type flies, whereas mutant flies were delayed regardless of whether their mothers were heterozygous or homozygous for the mutation. The mutant flies were also markedly temperature sensitive, showing relatively good survival to eclosion at 21° (total number of offspring from 90 females, 86% of that from wild type), but slightly less at 25° (74% of the wild type), and a low rate of successful completion of development at 16° (19% of the number of wild-type flies eclosing).

As expected for a mutant postulated to have a quantitative defect in the availability or function of mitochondrial ribosomes, the flies were hypersensitive to doxycyclin, an antibiotic that specifically inhibits mitochondrial as opposed to cytosolic translation (VAN DEN BOGERT *et al.* 1988). When raised in medium containing increasing doses of this drug (Figure 3), *tko*$^{25t}$ mutant flies showed much lower numbers surviving to eclosion than wild-type flies (Figure 3a) and exhibited an extended developmental delay (Figure 3b). Moreover, wild-type flies raised in medium containing 1 mg/ml doxycyclin exhibited a developmental delay similar to that of *tko*$^{25t}$ mutant flies (Figure 3b) and were also significantly bang sensitive (mean recovery time from vortex-induced paralysis was 12.6 ± 1.6 sec, based on 50 flies of each sex). Growth in doxycyclin therefore generates a partial phenocopy of the *tko* mutation and acts synergistically with it. These findings strongly support the view that both the developmental and sensorineural phenotypes of *tko*$^{25t}$ mutant flies are the result of a reduced capacity for mitochondrial protein synthesis.

In contrast, streptomycin, an antibiotic that acts by impairing translational accuracy in eubacterial ribosomes, had no significant effects on the development of wild-type or *tko*$^{25t}$ mutant flies at concentrations ≤1 mg/ml (data not shown). Wild-type mitochondrial ribosomes, like those of the cytosol, are resistant to this drug. The absence of any effect in the mutant indicates that the mutation is unlikely to affect the fidelity of

**TABLE 1**

**Courtship analysis of *tko*$^{25t}$ and wild-type flies**[a]

| Males | Females | No. of copulations ($N$ = 50) | Mean courtship time (min) ± SE | Mean copulation time (min) ± SE |
|---|---|---|---|---|
| Wild type | Wild type | 32 | 16.0 ± 2.1 | 30.3 ± 1.3 |
| Wild type | *tko*$^{25t}$ | 34 | 12.2 ± 2.4 | 26.5 ± 0.7 |
| *tko*$^{25t}$ | Wild type | 1 | (12.0)[b] | (21.0)[b] |
| *tko*$^{25t}$ | *tko*$^{25t}$ | 18 | 22.0 ± 4.1 | 19.0 ± 0.8 |

[a] All flies tested were 3 days old.
[b] Courtship and copulation time for the sole pair that mated.

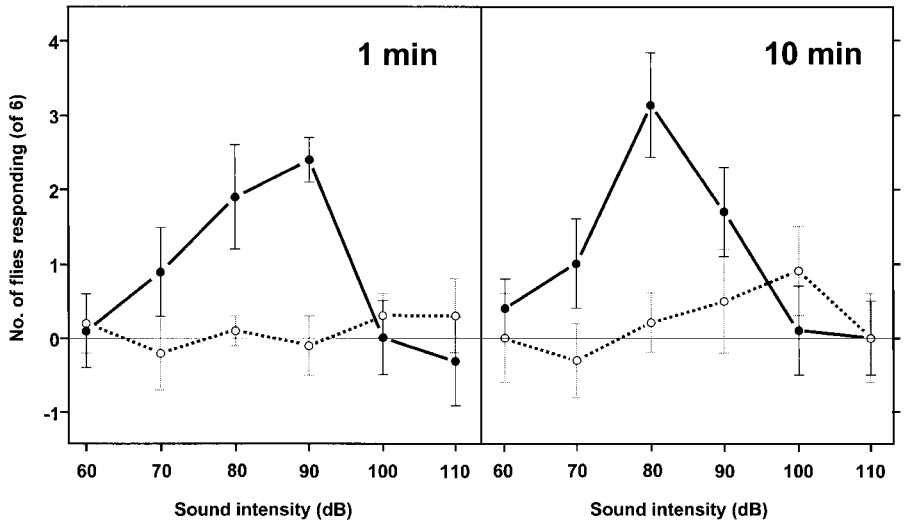


FIGURE 2.—Impaired sound responsiveness of *tko*$^{25t}$ mutant flies. Hearing was measured by the male courtship song response assay as described in the text. The mean number of flies (±SD), in batches of six, responding to courtship song played for 1 min and 10 min is plotted against sound intensity. Filled circles and solid lines, wild-type males; open circles and dotted lines, *tko*$^{25t}$ mutant males. Faint horizontal lines indicate the zero response level. Wild-type (hearing) flies show pronounced response at 70–90 dB even after 1 min, whereas mutant flies show only a minimal response at abnormally high volume (100 dB) over 10 min.

mitochondrial translation. The result is also consistent with modeling of the *tko*$^{25t}$ mutation in *E. coli* (TOIVONEN *et al.* 1999).

To confirm quantitative effects on mitochondrial function we analyzed mitochondrial redox activities in third instar larvae of wild-type and *tko*$^{25t}$ mutant flies (Figure 4). To control for any effect of inbreeding *per se*, we used, in addition to Canton-S, an inbred reference strain (0713) as a wild-type control. Large and significant decreases in complexes I, II, and IV were evident in *tko*$^{25t}$ larvae, compared with either reference strain (Figure 4a). Citrate synthase activities measured in the same extracts showed no significant differences between the strains. ATP synthesis capacity was less affected (Figure 4b), although the effects were still significant, at least for two of the substrate mixes tested. A clear effect on the level of small subunit (12S) compared with large subunit (16S) mitoribosomal RNA was also seen in third instar larvae (Figure 4c), indicating a relative deficiency of small subunits. Densitometry on replicate blots using different amounts of RNA indicated that the amount of 12S relative to 16S rRNA in the mutant was only 30% of that in wild-type flies. Use of a cDNA probe for a cytosolic ribosomal protein, rp49, as loading control showed that this was due to a deficiency of 12S rather than an excess of 16S rRNA (data not shown).

**Construction of *tko*$^{25t}$ H85L revertants by *P*-element transgenesis:** To test whether the complex phenotype exhibited by *tko*$^{25t}$ mutant flies is wholly attributable to the L85H substitution in the coding sequence of mitoribosomal protein S12 (mt-rps12), we constructed strains of transgenic flies carrying additional autosomal copies of the gene, which were derived originally from genomic DNA of the mutant strain, and which either had or had not been reverted to leucine at residue 85. The strategy by which this was accomplished is summarized in Figure 5 and detailed in MATERIALS AND METHODS. Briefly, the 3.2-kb *Bam*HI fragment containing the mt-rps12 coding sequence was cloned from genomic DNA of *tko*$^{25t}$ mutant flies via a high-fidelity PCR strategy and then subjected to PCR *in vitro* mutagenesis to correct the leucine-to-histidine substitution at residue 85. The structure of

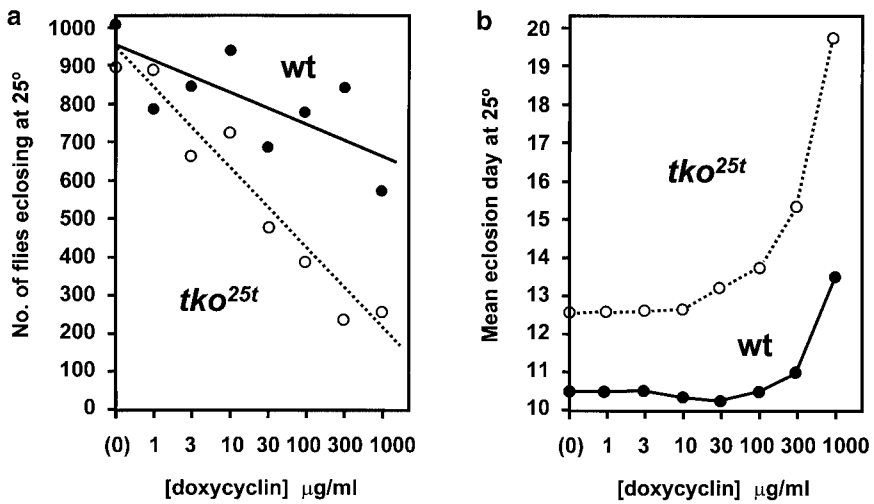


FIGURE 3.—Developmental delay and doxycyclin hypersensitivity of *tko*$^{25t}$ mutant flies. (a) Total numbers of flies eclosing after growth at 25° on medium containing increasing concentrations of doxycyclin, each data point representing the offspring from 30 females, set up in 5 replicate vials (6 females plus 3 males each). Filled circles, wild-type Canton-S; open circles, *tko*$^{25t}$. (b) Mean eclosion day of the same flies, plotted similarly, males and females pooled. The ratio of males and females eclosing was unaffected by the drug. SEs are omitted for clarity, but were all <0.1 days.

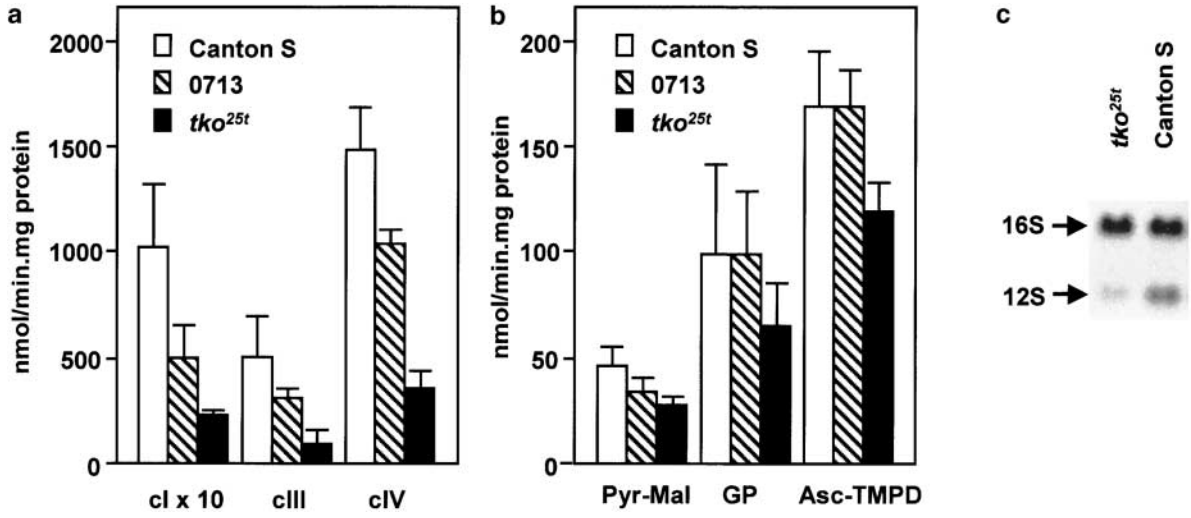


FIGURE 4.—Metabolic and rRNA analysis of *tko*$^{25t}$ mutant third instar larvae. (a) Enzymatic activities of mitochondria isolated from Canton-S, inbred wild-type (0713), and *tko*$^{25t}$ mutant larvae. cI, complex 1 (NADH:ubiquinone oxidoreductase); cIII, complex III (ubiquinol:cytochrome *c* oxidoreductase); cIV, complex IV (cytochrome *c* oxidase). Citrate synthase activities showed no significant differences between the strains. (b) ATP synthesis capacities of mitochondria isolated from the three strains, using the following different substrates: Pyr-Mal, pyruvate plus malate; GP, α-glycerophosphate; and Asc-TMPD, ascorbate plus TMPD. (c) Northern hybridization of RNA from third instar larvae of *tko*$^{25t}$ and Canton-S strains, probed simultaneously for mitochondrial 12S and 16S rRNA. Autoradiographic exposure time was 25 min.

all constructs was verified by DNA sequencing (see EMBL data library accession no. AJ250320). Both versions of the 3.2-kb fragment were cloned into the *P*-element vector pP{CaSpeR-4} and then independently micro-injected, together with a *P*-element transposase-encoding plasmid, into the germline of recipient *white* mutant embryos. Injected embryos that survived to adulthood were mated to *white* mutants, and putative transgene-containing, red-eyed progeny from the next generation were retained. Three transgenic lines (A1–A3) carrying different insertions of the unmanipulated *tko*$^{25t}$ mutant allele and eight transgenic lines (B1–B8) carrying different

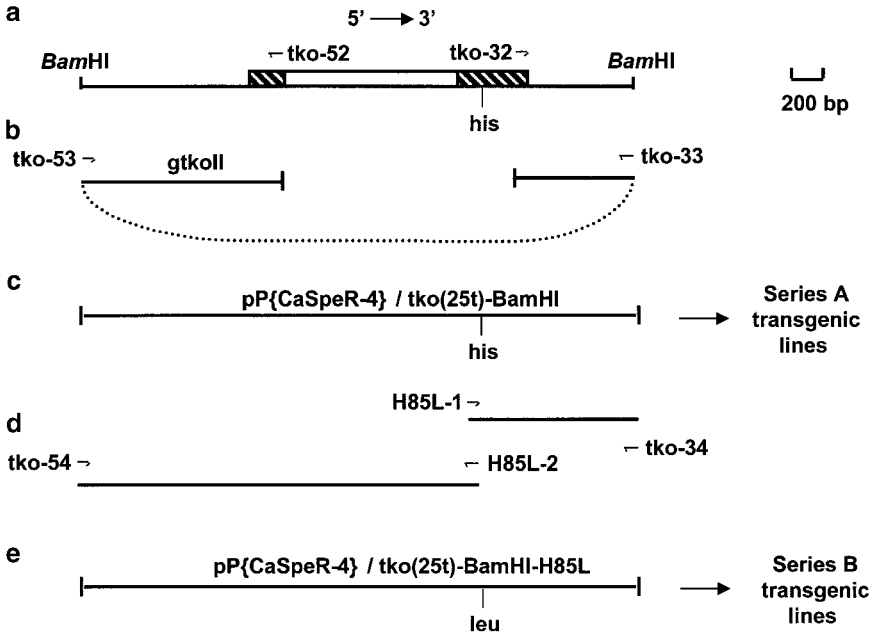


FIGURE 5.—Cloning/mutagenesis scheme for creation of transgenic flies. For full details refer to MATERIALS AND METHODS. (a) Schematic map of the 3.2-kb *Bam*HI fragment of genomic DNA from the mutant strain *tko*$^{25t}$. The two exons of the gene are shown as shaded boxes, and the single intron is shown as an open box, with the positions and orientations of primers tko-52 and tko-32 indicated. The mutant carries a C → A point mutation at codon 85 of the coding sequence, replacing a conserved leucine with histidine (see also Figure 6e). (b) Genomic DNA from the mutant strain was digested with *Bam*HI, circularized by ligation (dotted line), and amplified using primers tko-52 and tko-32 to generate the 1.7-kb "flanking" fragment cloned as gtkoII. (c) The entire 3.2-kb *Bam*HI fragment was cloned from *tko*$^{25t}$ genomic DNA using primers tko-53 and tko-33. The fragment was cloned directly into the *P*-element vector pP{CaSpeR-4} to generate the "mutant" construct pP{CaSpeR-4}/tko(25t)-*Bam*HI for micro-injection, from which transgenic lines A1–A3 were obtained. (d) A two-stage PCR *in vitro* mutagenesis strategy was used to create the replacement mutation at residue 85, restoring the wild-type decoding specificity for leucine. The products of the first step were mixed and reamplified using the terminal primers, then recloned into the vector to create e, the reverted construct pP{CaSpeR-4}/tko(25t)-*Bam*HI-H85L, from which transgenic lines B1–B8 were obtained. To introduce the Q116K substitution, an equivalent procedure was employed, starting from the *in vitro*-reverted construct (e). From the eventual construct pP{CaSpeR-4}/tko-*Bam*HI/*Eco*RI-Q116K transgenic lines Q116K(1), Q116K(3), and Q116K(6) were obtained.

insertions of the reverted (H85L) allele were obtained and each was tested for homozygous viability, for the chromosomal location of the insertion, and for the presence of an intact *tko* transgene at an ectopic site by PCR and Southern blotting.

All insertion(s) were autosomal, and all except line B8 (reverted allele) were homozygous viable. An intact transgene was found in every case by PCR (data not shown), and Southern analysis showed that most of the transgenic lines contained just one or two ectopic copies of the *tko* gene, although 1 line (A3) carried a number of additional ectopic copies of the unreverted allele (data not shown). All 11 transgenic lines, *i.e.*, the 8 transgenic lines carrying different autosomal insertions of the reverted (H85L) allele plus 3 carrying different autosomal insertions of the unmanipulated $tko^{25t}$ mutant allele, were initially characterized in a *tko* wild-type genetic background. None was bang sensitive, nor did any of them exhibit developmental delay or other features of the $tko^{25t}$ mutant phenotype, as expected, given the recessive nature of the mutation. No other phenotypic abnormalities were observed in the transgenic lines bred to homozygosity, except the lethality in line B8 already mentioned and a variegated eye-color phenotype in line B1.

**Phenotypic analysis of $tko^{25t}$ H85L revertants:** Males from the transgenic lines were mated to $tko^{25t}$ homozygous females (see scheme illustrated in Figure 6, a and b), and the progeny were scored initially for the developmental delay characteristic of the $tko^{25t}$ mutant. As expected, all female progeny were phenotypically wild type, since they each carried a wild-type copy of the *tko* gene on the X chromosome inherited from their transgenic fathers. The male progeny, which all carried the $tko^{25t}$ mutant allele on their X chromosome, showed a clear-cut restoration of wild-type developmental timing for all lines carrying an autosomal copy of the reverted allele (mean eclosion at 25° between 10.1 and 10.7 ± 0.1 days for all eight lines), whereas all three lines with one or more additional copies of the mutant allele showed the same 2- to 3-day developmental delay as the $tko^{25t}$ mutant itself, with eclosion at 25° at days 13.4 ± 0.1 for line A1 and 12.7 ± 0.1 for lines A2 and A3. Two simple conclusions emerge. First, the presence of an extra hemizygous dose (or several) of the $tko^{25t}$ mutant allele does not significantly complement the developmental timing defect. Second, the L85H substitution in the $tko^{25t}$ mutant allele can alone account for the developmental delay of the mutant.

Hemizygous lethality was tested by crossing males of each transgenic line with females heterozygous for a lethal allele of *tko* ($tko^{3}$) over the FM7 balancer (Eberl *et al.* 1997; see scheme shown in Figure 6, c and d) and scoring for eclosed adult males lacking the markers carried on the FM7 chromosome. The $tko^{3}$ allele (Lindsley and Zimm 1992) contains a short insertion/deletion at codon 108–109 (Figure 6f), resulting in a frameshift and premature stop, eliminating the C-terminal part of the protein, part of which is highly conserved in all rps12 homologues. $tko^{3}$ must therefore be a null allele, and all male progeny from the cross that lack the balancer must carry only this null allele on their X chromosome, plus one copy of either the reverted or the unreverted $tko^{25t}$ allele on an autosome. Male progeny from all eight reverted transgenic lines eclosed successfully, whereas $tko^{3}$ males carrying only the unreverted $tko^{25t}$ transgene did not. This applied even to line A3, carrying additional autosomal copies of the transgene. The L85H substitution is thus inferred to be the cause of hemizygous lethality in the $tko^{25t}$ mutant.

One unreverted transgenic line (A1) and three H85L-reverted transgenic lines (B1, B2, and B5) were studied in further detail. First, crosses were set up to reevaluate developmental timing in flies both hemizygous and homozygous for each transgene, in both sexes, in the $tko^{25t}$ mutant background. All $tko^{25t}$ flies carrying also the (unreverted) transgene from line A1 had a mean eclosion day of ~12.5, like the $tko^{25t}$ mutant itself. All $tko^{25t}$ flies carrying the reverted transgene from lines B1, B2, or B5 had a mean eclosion day of ~10, like wild-type flies. In the $tko^{25t}$ mutant background the reverted lines were no longer bang sensitive (Figure 7), and males carrying the reverted transgene from line B5 were able to mate with wild-type females at the same frequency and with similar timing as did wild-type males (Table 2). Flies carrying the unreverted A1 transgene in the same background were still bang sensitive (Figure 7) and also defective in male courtship (Table 2, refer also to Table 1), exhibiting prolonged courtship time, reduced copulation time, and a low frequency of successful mating, although the latter effect was somewhat less marked than for the original mutant line. Enzymatic analyses of larval mitochondria (data not shown) also indicated that the wild-type but not the mutant transgene restored mitochondrial redox enzymes to the levels of the wild-type strain. The L85H substitution therefore appears sufficient to account for all the main features of the mutant phenotype, whether metabolic, developmental, or behavioral.

**Phenotypic analysis of the Q116K substitution in *tko*:** Extensive previous analysis of the *tko* gene homologue in bacteria (*rpsL*) has given clear functional insight into different regions of the protein. In *E. coli*, the *rpsL* mutation L56H, equivalent to L85H in *tko*, gives a phenotype of impaired ribosome assembly, but without detectable effects on translational accuracy (Toivonen *et al.* 1999). In contrast, residue K87 in bacterial *rpsL* is the site of numerous mutations to aminoglycoside resistance and increased stringency. The analogous residue is substituted by glutamine in the mitochondrial *rpsL* homologue in all metazoans analyzed, a substitution that gives low-level streptomycin resistance in *E. coli* (Toivonen *et al.* 1999). We reasoned that "reverting" this residue to lysine in Drosophila *tko* should relax the stringency of mitochondrial translation, providing a test of whether

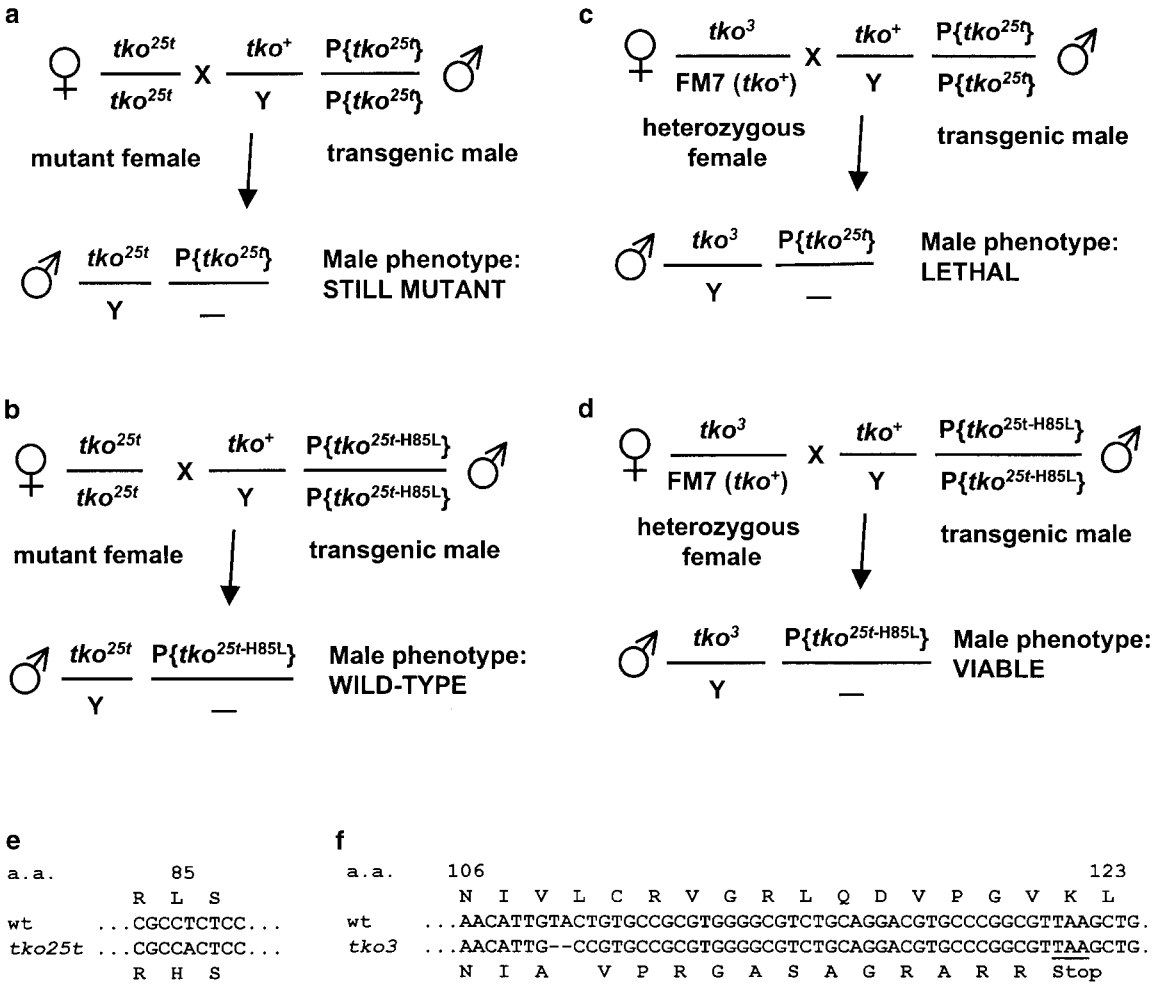


FIGURE 6.—Scheme for phenotypic analysis of transgenic lines. (a and b) Mating scheme to test effects of *tko* transgenes on developmental timing. (c and d) Mating scheme to test viability of flies hemizygous for *tko* transgenes. (e and f) Details of the mutations in the mt-rps12 coding sequence found in $tko^{25t}$ and $tko^{3}$ flies. Amino acids are numbered from the N-terminal methionine of the putative precursor protein. Gaps are indicted by dashes, and the premature stop codon created by the $tko^{3}$ mutation is underlined.

the complex phenotype of the original $tko^{25t}$ mutant is attributable to qualitative or quantitative defects in mitochondrial translation. We therefore constructed a version of *tko* in pP{CaSpeR-4} carrying this substitution, Q116K, and derived transgenic lines in a *tko* wild-type background.

Three independent lines, Q116K(1), Q116K(3), and Q116K(6), each carrying apparently single insertions of the Q116K transgene on the third, second, and third chromosomes, respectively, were bred to homozygosity for further analysis. All were initially viable and showed no developmental delay nor any other obvious phenotype. One additional transgenic line had an eye phenotype that is almost certainly attributable to an insertional effect and was not investigated further in this study. One line, Q116K(1), progressively lost viability as a homozygote, and in crosses to wild-type flies this was found to be a maternal effect. All three were crossed into the *tko*-null ($tko^{3}$) background and bred to homozygosity. Lines Q116K(3) and Q116K(6) remained viable in this state, although line Q116K(1) again lost viability after three to five generations. In crosses to wild-type males, all three lines were female sterile, although courtship appeared normal. The female sterility was marked by a failure to lay eggs. No other developmental abnormalities were observed. The flies showed no sensitivity to streptomycin (data not shown).

## DISCUSSION

This is the first demonstration that a mutation in an essential component of the mitochondrial translational apparatus gives rise to a whole-organism phenotype in a model metazoan. The phenotype has both developmental and behavioral aspects that reflect features of mitochondrial disease in humans, for which it represents a useful model.

**Developmental consequences of a mitochondrial**

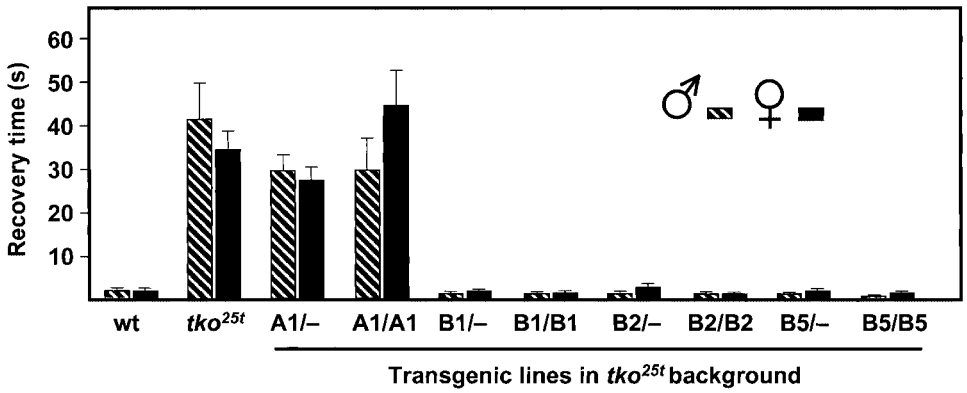


FIGURE 7.—Phenotypic analysis of transgenic flies. Recovery times from paralysis after 15 sec of vortexing (mean ± SE of measurements on 50 individual flies of each sex and genotype, all tested between 2–4 hr after eclosion). The following flies were tested: wild-type Canton-S; inbred $tko^{25t}$; and the transgenic derivatives A1 (mutant construct), B1, B2, and B5 (all revertant constructs) as hemizygotes (A1/– etc.) or homozygotes (A1/A1 etc.), always in a $tko^{25t}$ background.

**translational defect in Drosophila:** The developmental delay associated with the $tko^{25t}$ mutation is zygotically determined and entirely postembryonic, occurring mainly in the larval stages. This accords with the fact that mtDNA (KELLEY and LEE 1983) and its transcription/translation products (TOURMENTE *et al.* 1990; TALAMILLO *et al.* 1998) are accumulated during oogenesis in quantities that appear to be sufficient for embryonic development. There is no replication of mtDNA during embryogenesis (RUBENSTEIN *et al.* 1977), and mitochondrial transcripts fall to low levels (KELLEY and LEE 1983), indicating that new mitochondrial protein synthesis is unimportant during the period of rapid cell division, fate determination, and gastrulation. In conformity with this, null alleles of *tko*, such as $tko^{3}$, are larval lethals that nevertheless complete embryogenesis.

In contrast, the increased time taken by the $tko^{25t}$ mutant to complete the larval stages fits with the idea that zygotic expression of components of the mitochondrial translational apparatus is required during this phase of net growth. Mitochondrial energy limitations, as implied by the decreased level of mitochondrial redox enzymes containing mitochondrial translation products in mutant larvae, may constrain the rate of cell division in one or more cell types. The larval stages are dedicated to the increase of biomass based on the metabolization of food sources; hence, it is not surprising that a defect in the system that produces the bioenergy-generating complexes would limit the overall rate at which new biomass is accumulated.

Flies heterozygous for null alleles of *cytosolic* ribosomal protein genes (*e.g.*, RpL19, RpS3) generally show the characteristic minute (MIN) phenotype, also involving delayed eclosion and short, slender bristles (KONGSUWAN *et al.* 1985; LAWRENCE *et al.* 1986; KAY and JACOBS-LORENA 1987; SAEBOE-LARSSEN *et al.* 1998), but not bang sensitivity. The developmental delay has been attributed to an increased cell cycle time and the morphological phenotype to a requirement for maximal rates of protein synthesis for bristle elaboration. Although $tko^{25t}$ is not a MIN mutant, since it behaves as a true recessive and exhibits distinct behavioral features, the phenotypic overlap suggests strongly that efficient mitochondrial translation is also required for maximal rates of cell division. This conclusion is further strengthened by our finding that growth of flies on medium containing doxy-

**TABLE 2**

**Courtship analysis of transgenic flies**[a]

| Males | Females | No. of copulations ($N = 50$) | Mean courtship time (min) ± SE | Mean copulation time (min) ± SE |
|---|---|---|---|---|
| Wild type | A1/−[b] | 41 | 7.4 ± 1.0 | 23.5 ± 0.5 |
| Wild type | A1/A1 | 38 | 9.1 ± 1.4 | 23.6 ± 0.4 |
| Wild type | B5/− | 39 | 7.0 ± 1.2 | 23.2 ± 0.5 |
| Wild type | B5/B5 | 46 | 9.1 ± 1.2 | 23.8 ± 0.6 |
| A1/− | Wild type | 9 | 17.6 ± 2.8 | 16.8 ± 0.4 |
| A1/A1 | Wild type | 11 | 23.0 ± 2.9 | 15.9 ± 0.3 |
| B5/− | Wild type | 35 | 7.1 ± 0.9 | 21.7 ± 0.4 |
| B5/B5 | Wild type | 40 | 10.2 ± 1.6 | 24.0 ± 0.5 |

[a] All flies tested were 3 days old.

[b] A1/− denotes hemizygosity, A1/A1 denotes homozygosity for the (unreverted) autosomal transgene from line A1, in the $tko^{25t}$ mutant background, etc.

cyclin produces a very similar developmental delay to that seen in the *tko*$^{25t}$ mutant or indeed in MIN mutants.

**Behavioral consequences of a mitochondrial translational defect in Drosophila:** The *tko*$^{25t}$ mutant exhibits bang sensitivity, hyporeactivity, a defect in neuronal transmission from mechanoreceptor cells, and impaired response to sound. This raises a number of questions regarding the relationship of these phenotypes to each other and to the inferred deficit in mitochondrial translation.

Like deafness in vertebrates (Hardisty *et al.* 1998), bang sensitivity in Drosophila is a commonly observed phenotype. It is associated with mutations in genes involved in a variety of cellular structures or pathways. These include phospholipid biosynthesis, *e.g.*, *easily shocked*, encoding ethanolamine kinase (Pavlidis *et al.* 1994), ion transport, *e.g.*, *Atp*α, encoding the α-subunit of a plasma membrane Na+,K+-ATPase (Schubiger *et al.* 1994), and transcription, *e.g.*, *bang senseless*, encoding the HMG-domain-containing transcriptional modulator DSP1 (Kirov *et al.* 1996). One other bang-sensitive mutant, *stress sensitive B* (*sesB*), also has a mutation in a gene encoding a key accessory component of the apparatus of mitochondrial oxidative phosphorylation, namely one of two isogenes for the adenine nucleotide translocator (Zhang *et al.* 1997), which makes mitochondrially synthesized ATP available for other cellular processes. This supports the idea that the behavioral effects of *tko*$^{25t}$ result from mitochondrial energy insufficiency. Like *tko*, but unlike other bang-sensitive mutants tested, *e.g.*, *slamdance* and *easily shocked*, *sesB* also exhibits developmental delay (K. M. C. O'Dell, unpublished data). Two dominant, temperature-sensitive paralytic mutants, *Outcold* and *Third Cold Paralytic*, also appear to have defects in mitochondrial energy metabolism (Sondergaard *et al.* 1975; Sondergaard 1980, 1986; Pavlidis and Tanouye 1995; Walker *et al.* 2000).

Why does a failure in mechanoreceptor transmission result in bang sensitivity? The major bang-sensitive mutants, including *tko*, show a failure in the giant fiber pathway (Pavlidis *et al.* 1994; Pavlidis and Tanouye 1995). Electrical stimulation of the brain leads to seizures, involving failure at the synapse between a peripherally synapsing interneuron and motorneurons. Exactly how this is linked to failure of mechanoreceptor transmission is still unclear: input from mechanosensory neurons may be required to modulate the motor response. Since the strength of the *tko*$^{25t}$ bang-sensitive phenotype declines rather rapidly after eclosion and is also weaker in the inbred line, some kind of physiological adaptation to the mechanosensory defect seems to occur. It will be interesting to study this by crossing *tko*$^{25t}$ into other backgrounds with defined genetic defects in neural or neuromuscular function as well as in combination with other bang-sensitive mutations.

Why does a mitochondrial translational defect cause mechanosensory failure and impaired response to sound? Previous electrophysiological measurements of *tko*$^{25t}$ flies indicated that the frequency of action potentials in response to a bristle displacement was reduced (Engel and Wu 1994). Mitochondrial energy may be needed for one or more of several different steps in this process. The primary step in insect mechanoreceptor activation is the opening of a mechanically gated ion channel (Walker *et al.* 2000) as in nematodes (Tavernarakis and Driscoll 1997) and the vertebrate inner ear (Denk *et al.* 1995). Reclosing of the channel may be hypothesized to involve cytoskeletal remodeling, possibly coupled to a series of ionic changes, either one or both of which may be mitochondrially energized. The transduction of ionic currents across the receptor cell, the movement or membrane fusion of synaptic vesicles, the reuptake of a neurotransmitter, or the recycling of ions to enable the system to fire successively may all be dependent on respiratory energy. For example, mitochondria are believed to have a particularly crucial role in calcium pumping (Ichas *et al.* 1997; Jouaville *et al.* 1998). Whether the impaired response to sound is directly or solely caused by defective mechanotransduction remains to be established; for example, it could also involve abnormalities in central processing.

The behavioral phenotype of *tko*$^{25t}$ flies extends beyond bang sensitivity and impaired response to sound to include decreased reactivity and defective courtship. The courtship defect fits with a primary physiological impairment in sound perception, although other sensory systems may also be affected. Courtship involves recognition of sensory cues by both sexes, including visual and olfactory stimuli as well as sound (see Hall 1994 for review). A crucial step is the female's interpretation of the male courtship song, composed of a complex, species-specific, wing-beating behavior. Female discrimination of fine details of the courtship song is crucial to mating success (Ritchie *et al.* 1998) and is a primary determinant of the rejection of even sibling species (Tomaru *et al.* 1995).

The male courtship defect of the *tko*$^{25t}$ mutant may have more than one component and is perhaps partly attributable to the reactivity deficit. However, the mutant male's courtship behavior is apparently not perceived as inadequate by mutant females, although this could be influenced by selection during inbreeding, as observed for the *raised* (*rsd*) mutant (McRobert *et al.* 1995). The defect manifests in a similar way to that of a number of other mutants that were selected initially in a screen for flies with impaired auditory perception (Eberl *et al.* 1997), notably *acd(2)5L3* and *btv* (*beethoven*, 5P1 allele). These mutant males, like *tko*$^{25t}$, were also relatively unsuccessful in copulating with wild-type females, suggesting that a defect in sound perception in some way impairs the production of courtship song or other courtship behavior. The absence of a rejection response from *tko*$^{25t}$ females may simply indicate that

they fail to distinguish the mutant male's song as abnormal, due to their own sensory defect. To test these ideas will require high-resolution video time-lapse and sound recording of the mutant male's courtship song and of both wild-type and mutant females' response to it.

**Quantitative effects of the *tko$^{25t}$* mutation:** As in *E. coli*, where the equivalent mutation (L56H) in *rpsL* caused a severe defect in ribosome assembly (Toivonen *et al.* 1999), the *tko$^{25t}$* mutation results in a functional deficiency of mitochondrial ribosomes, as evidenced by decreased levels of 12S rRNA and the diminished amounts of mitochondrial redox enzymes dependent on mitochondrial translation products. Since ribosomal RNA is unstable if not incorporated into ribosomal subunits (Dennis and Young 1975), the low levels of 12S rRNA in the mutant indicate a corresponding 70% decrease in the amount of mitoribosomal small subunits and must reflect either a defect in assembly or stability of ribosomes. The fact that this provokes a corresponding deficiency in the activities of complexes I, III, and IV in third instar larvae suggests that in the larval stages, mitochondrial protein synthesis is limiting for redox enzyme assembly, and that mitoribosome availability is just sufficient to maintain the required level of protein synthesis.

However, the presence of an additional copy of the mutant allele on an autosome did not detectably improve the mutant phenotype. This strongly implies that the mutant phenotype is not simply related to gene dosage, although female hemizygous lethality implies that, if the expression of the mutant allele falls below a critical threshold, normal development cannot be completed. Male flies hemizygous for an autosomally located transgenic copy of the mutant allele also did not eclose. In fact even line A3, which carries at least three autosomal copies of the unreverted transgene, did not rescue the null mutant. Therefore, the information for full expression and/or dosage compensation of the gene cannot be carried on the 3.2-kb fragment used for transgenesis. Expression analysis by cDNA minisequencing (S. Manjiry and J. M. Toivonen, unpublished data) confirmed that transgene expression was typically ~30% that of the endogenous gene. In contrast, flies hemizygous for a single autosomal copy of the reverted *tko$^{25t}$* allele were phenotypically wild type even in the *tko$^{3}$* background, regardless of sex. Therefore, even suboptimal expression of the wild-type allele is sufficient to restore wild-type phenotype, which is fully consistent with the recessive nature of the *tko$^{25t}$* mutation.

**The *tko* fly as a model for human mitochondrial disease:** We set out initially to test whether a point mutation engendering a nonconservative amino acid substitution in the coding region of mitoribosomal protein S12 could account for the bang sensitivity of the *tko$^{25t}$* mutant, which was postulated as a model for mitochondrial deafness. Our findings strongly support this conclusion: reversion of the mutation abolishes the behavioral as well as the developmental phenotype of the mutant. Moreover, the mutation increases the sensitivity of the flies to the effects of a mitochondrial translational inhibitor. In that sense it resembles at least one mitochondrial mutation in humans, at nucleotide pair 1555, which sensitizes carriers to aminoglycoside ototoxicity (Prezant *et al.* 1993).

Most of the gene products involved in mechanoreceptor function are not highly conserved at the sequence level, although it has recently been argued that, because insect mechanoreceptors and vertebrate inner-ear hair cells are developmentally related, they are likely to have a common evolutionary origin (Eberl 1999) and to operate in a similar way at the molecular level. It is striking, therefore, that mitochondrial translational dysfunction should compromise mechanoreceptor function and hearing in both flies and humans. Mitochondrial mutations affecting the translational apparatus are associated with both syndromic and nonsyndromic forms of human sensorineural deafness (Jacobs 1997; Fischel-Ghodsian 1999). In no case, however, is it understood how a mitochondrial translational defect produces this tissue-specific disorder. Even the precise cell type(s) affected have not been characterized. The availability of a model system, such as that afforded by *tko*, in which the cell biology, physiology, and developmental aspects of the defect may be studied directly, offers great promise in understanding the pathogenesis of mitochondrial deafness, especially given that the mechanosensory apparatus in this organism is physically accessible for experimentation.

The developmental effects of the *tko$^{25t}$* mutation may be relevant to other aspects of mitochondrial disease. The most common inherited pathological point mutation in mtDNA in humans, A3243G, has a complex and variable phenotype (Fadic and Johns 1996). A recent epidemiological survey concluded that the most frequently observed clinical features included both hearing impairment and short stature (Majamaa *et al.* 1998). A slowed rate of growth or of cell division during critical phases of development, resulting from limitations brought about by mitochondrial translational insufficiency, may therefore apply in both flies and humans. The female sterility resulting from the Q116K mutation in *tko* was unexpected and warrants further investigation. Both of the mutant alleles of *tko* studied here may provide exploitable mitochondrial disease models in the fly.

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