Molec. gen. Genet. 120, 107—114 (1973)
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Temperature-Sensitive Mutations in *Drosophila melanogaster*

XIV. A Selection of Immobile Adults *

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Received September 26, 1972

Summary. Improved methods for rearing and screening large numbers of flies permitted the recovery of 10 mutations exhibiting a reversible temperature-dependent adult paralysis among 1.1×10^6 flies tested. Of the 10 mutations, two were allelic to $para^{ts}$, two were alleles in a new locus, stoned *(stn)*, and six fell into a third area, the shibire *(shi)* locus. Several of the *shi* alleles cause embryonic, larval and adult paralysis at 29° C as well as structural anomalies of various tissues. In addition to the ts mutations, several non-conditional mutations affecting adult movement were recovered.

Introduction

Mutations affecting a variety of "behavioural" characteristics of *Drosophila melanogaster* are being investigated in a number of laboratories. These studies include response to light (Hotta and Benzer, 1969; Pak *et al.*, 1969), leg and wing movement while under ether anesthesis (Ikeda and Kaplan, 1970), and diurnal rhythm (Konopka and Benzer, 1971). In order to screen for mutations possibly affecting nervous transmission and/or structural defects in musculature, we chose the phenotype of paralysis of adult flies as the criterion for selection. A defect in nervous or muscle tissue which results in paralysis obviously would prevent propagation of the genotype by the afflicted individuals. Consequently, we looked for mutations whose expression of a paralytic phenotype was temperature-dependent and reversible. The utility of temperature-sensitive mutations in a variety of studies in Drosophila has been demonstrated (Suzuki, 1970) and an initial study yielded one temperature-sensitive (ts) mutation out of 2.5×10^5 flies tested which caused paralysis (Suzuki *et al.*, 1971). The mutation, $para^{ts}$, produces instantaneous adult paralysis at 29° C which is immediately reversed upon shifting the flies back to 22° C. Anatomical studies of somatic mosaics strongly indicate a defect in the central nervous system (Grigliatti *et al.*, 1972). We have subsequently screened another 1.1×10^6 progeny of mutagenized parents for additional ts paralytic mutations and report here on the mutants recovered and their genetic and phenotypic properties.

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* This work was supported by the National Research Council of Canada, grant A-1764, National Cancer Institute of Canada, grant 6051.

** Medical Research Council of Canada Postdoctoral Fellow.

Materials and Methods

General Screening Protocol. The alkylating agent, ethyl methanesulfonate (EMS), is a potent mutagen when fed to Drosophila and induces a high frequency of ts mutations which are presumed to be missense alterations (Suzuki *et al.*, 1967; Suzuki, 1970). Wild-type males of an Oregon-R strain were fed 0.025 M EMS dissolved in 1% sucrose (Lewis and Bacher, 1968) for 24 hours at 22° C. The treated males were then mated to females carrying an attached-X chromosome (designated *C(1) DX*) at 22° C on standard Drosophila medium. Since F_1 sons of such flies receive a mutagenized paternal X chromosome, induced sex-linked recessive as well as autosomal dominant mutations could be detected. All adult F_1 progeny were then placed in a preheated plastic box at 29° C ($1.5–2 \times 10^4$ flies per test) and left for 1/2 to 2 hours. Separation of immobile flies from the rest of the F_1 population was accomplished by the entrapment of the paralyzed flies on a ledge while the mobile flies were drowned in a solution of detergent and vinegar (Williamson, 1971). Ready separation of putative paralytic flies was possible in a few minutes. The flies thus retained were then returned to 22° C and placed in vials. If they recovered mobility, males were mated to virgin *C(1)DX* females and females to wild-type males at 22° C. Their offspring were tested as adults for paralysis at 29° C.

The previous study (Suzuki *et al.*, 1971) required considerable time and effort in rearing and sexing parents and collecting F_1 progeny. Consequently, in order to minimize the logistics of screening large numbers of flies, several modifications were made in the procedure.

Screening Procedures. The mutation, *l (1)E6*^{ts}, is an allele of *ras* (for a detailed description of the mutations and chromosomes used, consult Lindsley and Grell 1968) which causes death when mutant flies are exposed to 28° C during the late larval and early pupal period (Grigliatti and Suzuki, 1970). Stocks containing this mutation were synthesized to provide a means of selectively killing either females or males, thereby yielding only adult males or virgin females and eliminating the need to sex flies by anesthesis and visual inspection.The female-producing stock utilized females carrying the compound-X chromosome, *C(1)DX*, mated to *l (1)E6*^{ts}/Y males. At 22° C, the stock remains stable and fertile. When virgin females were required, the parents were removed from the culture bottles 4–5 days after they were introduced and the bottles were shifted to 28° C where all *l (1)E6*^{ts}/Y males died so that only fertile *C(1)DX*/Y females hatched.

For the male-producing stock, an attached-X chromosome *(C(1)RM)* homozygous for *l (1)E6*^{ts} was constructed. *C(1)RM, E6*^{ts}/Y females were propagated at 22° C by mating them with wild-type males. When males were required, adults were removed from the culture bottles 4–5 days after their initiation and the bottles were shifted to 28° C where only wild-type males hatched. These males were then used for mutagenizing.

In this manner, $5–6 \times 10^4$ virgin females and males were collected at one time, the males were mutagenized and all were then introduced into a plexiglass box ($2' \times 2' \times 1.5'$) with a cheesecloth sleeve for insertion and removal of food. Approximately 10^4 new adults were introduced into the box weekly. Metal icecube trays containing a slab of Drosophila medium were then introduced into the box for 12 hours (16–20 trays at a time). Thousands of eggs were deposited on each tray which were then placed into shelved boxes with a cheesecloth sleeve (16–20 trays per box).

A thick paste of yeast was spread on each icecube tray daily to feed the larvae during the first 7 days of development. After 14–16 days, the trays were removed from the box. CO_2 was forced into the box through a small opening in order to anesthetize the flies. A trap door on the bottom of the box was then opened and the flies collected in flasks. In this manner, $2–5.5 \times 10^4$ adults could be collected per box. The flies were then weighed in order to estimate their number (an average of 1 mg/fly) and tested for paralysis at 29° C as outlined above.

Genetic Localization. Those lines producing a hereditary defect in mobility were retained and homozygous lines derived. Genetic localization of the sex-linked mutations was made relative to the markers (followed by their symbols and genetic positions) (Lindsley and Grell, 1968): yellow—*y* 0.0; crossveinless—*cv* 13.7; vermilion—*v* 33.0; forked—*f* 56.7; carnation—*car* 62.5, as was previously done for the mutation, *para*^{ts} (Suzuki *et al.*, 1971).

Results

An estimated total of 1.1×10^6 adult flies was screened. All but one of the mutations tested were sex-linked. Since EMS treatment induces a high rate of lethal mutations, the ratio of males to females was very low (0.3). Consequently, the actual rate of mutation induction on the X chromosome was much higher than the overall totals would indicate.

The selection technique at 29° C allowed not only the efficient recovery of ts immobilized flies, it also screened less efficiently for non-ts adults whose movement was impaired or retarded for any reason. A brief description of the latter group of mutations will be presented first.

Non-Temperature-Sensitive Mutations. Several mutations which appeared to fix the wings in set positions were recovered (Table 1). Thus, different mutations with wings held perpendicular to or on a 45° angle from the body were detected. Such flies could only walk or hop and therefore were trapped on the ledge of the screening box. These strains are being studied further by T. Grigliatti.

A dominant autosomal mutation which caused extreme debilitation of the posterior part of the adult was recovered in a fertile female. Flies carrying the mutation were extremely weak, reluctant (incapable) to mate and poorly fertile and the stock was lost after several generations. Segregation of the mutation verified its autosomal dominant nature and the phenotype was a dragging of the abdomen and the hind pair of legs.

When adults carrying the mutation *rex* (rapid exhaustion) are induced to move rapidly by constantly shaking them to the bottom of a vial or rotating a vial so that they continuously climb upwards, they fall to the bottom, paralyzed within 20 seconds. Paralysis lasts for about 30–40 seconds after which the flies stumble about and attempt to climb in an unco-ordinated fashion. Recovery rapidly occurs and the flies are then immune from paralysis for at least an hour. The mutation is a sex-linked recessive which maps genetically between *cv* and *v* at 17.0. The brevity of the paralytic state precludes easy study of the mutation.

Flies carrying the *bas* (bang-sensitive) mutation are immobilized by striking the culture vial on a hard surface. Paralysis persists for 30–40 seconds at which time the flies jump up and are immune to further paralysis for at least an hour. Phenotypically, this sensitivity to mechanical stress resembles the effects of *l(1)tko* located at the tip of the X chromosome (Judd *et al.*, 1972). F_1 females of the cross *bas*/Y♂ × *l(1)tko*/*l(1)tko*♀ are wild-type, thereby showing that the two mutant genes are not alleles. The locus was mapped between *v* and *f* at 47.2. Like *rex*, *bas* will be a difficult locus to study.

Adult *wob* (wobbly) flies cannot co-ordinate the proper sequence of leg movements for normal walking. Consequently, legs on one side of the body become entangled with the ones behind or in front. The legs of *wob* flies are held out very flat on a horizontal surface and the flies cannot easily climb or hang upside down on glass surfaces. Males are weak and very poorly fertile, undoubtedly due to an inability to mate. In crosses of *wob*/+ females to *wob*/Y males, homozygous *wob*/*wob* females have never been recovered as adults. It will be interesting to determine whether the lack of proper coordination results from weakened structural elements such as muscles in the leg or an altered hook-up of the nervous components co-ordinating the sequence of legs moved.

Table 1. Summary of the mutations recovered in screening for temperature-sensitive paralysis of adults

Locus	Genetic location	General phenotype at 29°C
	Non-temperature-sensitive	
several	X (not yet studied)	wings held at different angles from the body
unnamed (lost)	autosomes	dominant, abdomen and rear pair of legs dragged
rapid exhaustion (*rex*)	X (*cv-v* at 17.0)	temporarily paralyzed after rapid movement of the fly
bang-sensitive (*bas*)	X (*v-f* at 47.2)	temporarily paralyzed by mechanical shock on fly
wobbly (*wob*)	T (X→2R→3R→X)	legs weak and unco-ordinated, a leg often crosses one in front or rear of it
	Temperature-sensitive	
paralytic^{ts} (*para*^{ts})	X (*v-f* at 54.1)	only adults affected, immediate paralysis, instant recovery at 22°C
stoned^{ts} (*stn*^{ts})	X (*car-sfa* at 66.3)	unco-ordinated wing and leg movement
shibire^{ts} (*shi*^{ts})	X (*sd-f*) at 52.2)	adult paralysis, some alleles cause larval paralysis and egg lethality

Table 2. Properties of alleles of the shibire locus

Allele	Phenotype at 29°C
shi$^{ts\,1}$	embryonic lethal, larval and adult paralysis
shi$^{ts\,2}$	transitory larval paralysis, adult paralysis
shi$^{ts\,3}$	embryonic lethal, larval and adult paralysis
shi$^{ts\,4}$	only adult paralysis, adult *shi*$^{ts\,4}$/*shi*$^{ts\,1}$ and *shi*$^{ts\,4}$/shi$^{ts\,2}$ females paralyzed
shi$^{ts\,5}$	only adult paralysis, adult *shi*$^{ts\,5}$/*shi*$^{ts\,1}$ females paralyzed *shi*$^{ts\,5}$/*shi*$^{ts\,2}$ are not
shi$^{ts\,6}$	embryonic lethal, larval and adult paralysis

Genetic mapping of *wob* revealed an absence of crossing over between *y* and *cv* and a markedly reduced value for the *cv* to *v* region (11.0–12.0 compared with a normal value of 19.3). Cytological inspection of salivary gland chromosomes of the *wob* stock by T. C. Kaufman showed that the *wob* chromosome is, in fact, a translocation between the X, second and third chromosomes. The chromosome is designated as T(X→2R→3R→X) (Lefevre, 1972). EMS can clearly induce chromosome rearrangements, yet all EMS-induced ts mutations thus far mapped

behave as point mutants. Thus, selection for ts mutations may stringently select for missense defects.

Temperature-Sensitive Paralytics. Ten lines were recovered which yielded a hereditary temperature-dependent paralytic phenotype. The ten mutations in complementation tests with $para^{ts1}$ and each other, revealed at least three different complementation groups.

Two of the mutations in combination with $para^{ts1}$ yielded females which were immediately paralyzed at 29° C. Therefore, they were named $para^{ts2}$ and $para^{ts3}$. The *para* locus had been shown (Suzuki *et al.*, 1971) to map 2.6 units to the left of *f*. The phenotypic properties of these two alleles are indicated in Table 1. Unlike $para^{ts1}$-bearing flies, $para^{ts2}$ individuals are never completely paralyzed at 29° C and continue to exhibit leg kicking. Recovery of walking ability in $para^{ts2}$ flies at 29° C takes longer than in either $para^{ts1}$ or $para^{ts3}$ individuals. The allele, $para^{ts3}$, is the least extreme of the three in its phenotype at 29° C. Flies shifted to 29° C are quickly debilitated but continue to kick legs and stand and even walk in an unco-ordinated fashion. Walking ability at 29° C is recovered most rapidly by $para^{ts3}$ flies. Since each allele differs from the other in the extent of its paralysis, it is assumed that they represent genuine independently-induced mutations.

Two mutations failed to complement with each other but complemented with the other 8 ts paralytic mutations and therefore represent another locus. The locus was named stoned-temperature-sensitive (stn^{ts}). At 22° C, stn^{ts1} flies are visibly mutant and can be seen to walk slowly and carefully, occasionally flipping on their backs and fluttering their wings, although they can climb and fly. At 29° C, stn^{ts1} flies immediately fall to the bottom of the vial. They continue to kick while on their backs and never become completely immobilized. Nevertheless, their debilitation at 29° C is obvious and reversible at 22° C. Debilitation becomes more pronounced by longer (15 minutes or more) exposure to 29° C, although complete paralysis never occurs.

Flies carrying stn^{ts2} are quite active at 22° C. At 29° C, they are immediately rendered unable to climb the sides of the vials but continue to walk with a stiffened, stilt-like gait, stand upright and flutter their wings. Mobility is immediately recovered on shifting to 22° C. When stn^{ts2} flies are raised at 29° C from the egg stage to eclosion, the adults hatch but are permanently disabled. Upon shifting from 29° C to 22° C, such adults never climb the vials, walk in a stilted fashion and become stuck in the medium in a day or two. The *stn* locus maps genetically between the centromere and carnation at 66.3.

Six of the mutations appear to be non-allelic with the *para* and *stn* mutations but fail to complement with each other, and map between *v* and *f*, 0.7 units to the right of *sd*. The locus was named shibire (a Japanese word meaning paralyzed) and the alleles symbolized as shi^{ts1} to shi^{ts6}. The shi^{ts} alleles differ among each other in their effects on paralysis at 29° C (Table 2). At 22° C, shi^{ts} flies are indistinguishable from wild-type in walking, climbing and flying behaviour. Upon shifting to 29° C, the flies are immediately incapacitated in their climbing and flying ability. Complete paralysis requires over two minutes and then with extreme alleles such as shi^{ts1}, is complete for over 12 hours, at which time death occurs. At any time prior to this, flies shifted back to 22° C recover mobility within 3 minutes. The

sequence of events leading to paralysis upon shifting to 29° C, appears to be constant and repeatable:

0–20 seconds: unable to climb, stand normally or circle about; on bottom of vial.

20–40 seconds: legs extend, flies walk in stilt fashion and fall on backs.

40–60 seconds: very rapid movement of legs and wing flicking causing flies to flip about.

60–90 seconds: abdominal flexion, legs contracted, tarsi twitching, wings occasionally flick.

90–120 seconds: become progressively more paralyzed.

Recovery of mobility upon shifting paralyzed shi^{ts} flies from 29° C to 22° C is quite different. The flies lie on the bottom completely immobile. For some alleles, recovery occurs within 30 seconds, for others, after 2.5 minutes. The flies lie completely paralyzed, then suddenly flip upright and begin to walk. When the first flies show recovery, a tap on the vial will induce immediate recovery of all the others.

The first *shi* allele recovered, shi^{ts1}, produces complete adult paralysis within 3 minutes of shifting from 22° to 29° C. Paralysis persists until death 12–14 hours later. Adult shi^{ts1} flies shifted from 22° to 25° C are gradually debilitated and become completely paralyzed within 12 hours. Larvae of all developmental stages of shi^{ts1} also become paralyzed within minutes of a shift to 29° C. The larvae are immobilized in a characteristically elongated state and can be induced to contract posteriorly when the front end is probed. However, they do not turn aside in the normal avoidance reaction to the probe, nor are they sensitive to probes laterally or posteriorly. Eggs and pupae of the shi^{ts1} stock fail to hatch when shifted to 29° C. Two other alleles, shi^{ts3} and shi^{ts6}, also exhibit egg lethality and continuous larval and adult paralysis at 29° C (Poodry *et al.*, 1973).

The shi^{ts2} allele also produces prolonged adult paralysis. However, while the larvae are sluggish, they retain considerable movement after being shifted and developed to eclosion at 29° C, a striking difference from shi^{ts1} flies. Heterozygous shi^{ts1}/shi^{ts2} females exhibit the phenotype of shi^{ts1}.

The alleles, shi^{ts4} and shi^{ts5} produce adult paralysis at 29° C but larvae are not visibly affected by this temperature. However, even with prolonged maintenance of 29° C (longer than 30 minutes), both shi^{ts4} and shi^{ts5} adults are never completely motionless. Abdominal flexions continue and upon shifting back to 22° C recovery is rapid and complete within 30–45 seconds.

Discussion

This report demonstrates that selection of Drosophila adults on the basis of a phenotype of paralysis or reduced mobility yields a variety of mutations that may represent lesions in neural or muscular tissue. The utility of the property of temperature-dependent paralysis of the ts mutations which provides a simple environmental control of the mutant phenotype has already been demonstrated for a variety of studies (Suzuki *et al.*, 1971).

The most striking result of this study was the recovery of six alleles of the shibire locus. Multiple periods of ts lethality extending from the embryonic through

the adult stages, as well as a variety of temperature-induced abnormalities in the eye, bristles, wings, legs and tergites, have been demonstrated for shi^{ts1} (Poodry *et al.*, 1973). This points to a vital role of the *shi* locus. Moreover, the differences in the phenotypic effects and developmental stages affected by different *shi* alleles suggest a complex locus of considerable interest.

In addition to reversible ts paralysis, ts mutations affecting behaviour are being reported by other investigators. A mutation causing a reversible temperature-dependent abnormal electroretinogram has recently been discovered (M. Deland—personal communication). T. R. F. Wright (personal communication) has recovered a number of ts mutations affecting early embryonic muscle development. In a study of ts lethal mutations on chromosome 3, two mutations which affect adult leg movement have been recovered (E. Tasaka—personal communication). Both have a temperature-sensitive period in the pupal stage during which 29° C induces an irreversible effect on leg movement.

While stocks providing unisexual progeny and methods for culturing and screening relatively large numbers of flies facilitated the search and the yield of mutations, further improvements are still possible. The rate of recovery of ts paralytic mutations (1×10^5) is deceptively low. All of the ts mutations detected were recessives on the X chromosome, thereby suggesting that autosomal dominants do not occur or are too rare to merit consideration. Owing to the high level of induction of sex-linked lethals by EMS, crosses of mutagenized males to attached-X-bearing females yield male and female progeny in a ratio of about 1:2 or 1:3 (Baillie *et al.*, 1968). Thus, the yield of ts paralytics is, in fact, at least 2 or 3 times higher than the totals would indicate. Since F_1 females are of no value in screening for sex-linked recessives, a simple modification can eliminate them. Thus, the male-producing strain could be kept as $C(1)RM,\ l(1)E6^{ts}/Y^{bb^l}$ ♀ × $+/Y^{bb^l}$ ♂. Since the X chromosomes in both males and females carry bb^+, the flies are viable. On the other hand, $C(1)DX$, is deficient for the *bb* locus and therefore $C(1)DX/Y^{bb^l}$ females are inviable. Thus, mutagenized $+/Y^{bb^l}$ males when crossed to $C(1)DX/Y$ females would yield only males carrying mutagenized paternal X chromosomes. Further modifications such as longer periods of time at 29° C prior to selection should yield new paralytic sites.

It is becoming clear that new kinds of mutations exhibiting behavioural defects can be recovered if the proper phenotype is selected for and sufficient numbers can be efficiently screened. The potential information to be derived from such mutations is already indicated by the studies of $para^{ts}$ and shi^{ts}.

Acknowledgements. The capable technical assistance of Miss Anne Junker in the rearing and screening of flies is greated appreciated. Stimulating discussions with Dr. Thomas Kaufman and Dr. Clifton Poodry also assisted in the completion of the experiments.

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Communicated by Ch. Auerbach

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