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MOLECULAR GENETIC ANALYSIS OF THE CHEMOSENSORY SYSTEM OF *DROSOPHILA MELANOGASTER*

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INTRODUCTION

Chemosensory ability plays essential roles in the survival of insects: finding food source, avoiding predators, finding mate, and communications among peers. Although olfaction (smell) and gustation (taste) have been the subject of intense studies for many years, the molecular mechanism is still not well understood, and the various molecular components involved in chemosenses is only beginning to be identified in the past few years.

The dipteran fruitfly *Drosophila melanogaster* is an excellent experimental organism for studying the chemical senses for the following reasons. (a) It exhibits stereotyped behaviors, which can be easily measured quantitatively, in response to olfactory and gustatory stimulations. (b) The chemosensory organs are easily accessible. Sensitive electrophysiological measurements can be performed on them. The antennae, intact with their sensory hairs, can be isolated to high purity in modest quantity, thus facilitating biochemical and molecular biological studies. (c) The genetics of *Drosophila* is highly developed, making possible many useful genetic manipulations. (d) Olfactory and gustatory mutants have been isolated. (e) The availability of powerful molecular biology techniques, some unique to *Drosophila*, allow the cloning of almost any gene once identified by mutation. Once cloned, the gene can be manipulated *in vitro* and introduced back into the *Drosophila* germline and its *in vivo* activity be studied. Use of *Drosophila* in the genetic analysis of neurobiology has been very fruitful (Hall & Greenspan, 1979).

THE *DROSOPHILA* CHEMOSENSORY SYSTEM

Drosophila constructs their chemosensory system twice in development. The larval peripheral nervous system is histolyzed at the end of the larval stage, and during metamorphosis a new adult peripheral system is assembled to replace

the larval system; the central nervous system also undergoes substantial reorganization as well (Kankel *et al.*, 1980). As described later, all existing *olf* mutants exhibits the mutant phenotype at both the adult and larval stages, suggesting that these genes are being used in both system.

Antenna as the major olfactory organ

The primary olfactory organs in adult fly are located on the third segment of the antennae (Barrows, 1907). This segment is covered with approximately 300 innervated hairs or sensilla, consisting of three morphological types, basiconica, coeloconica and trichodea, of which the former two have been shown to have olfactory function. Each class of sensilla is distributed in a characteristic pattern (Venkatesh & Singh, 1984). At the base of each sensillum lie 2 to 4 bipolar olfactory neurons. Their dendrites extend into the lumen of the sensillum. Odorant molecules presumably come through pores on the sensillum wall and diffuse through the sensillar lymph before they reach the dendritic membrane, where is assumed to carry the receptor molecules.

Sensilla basiconica are also found on the maxillary palp, a part of the mouthparts of the fly. The sensory neurons in them project, via the labial nerve, to at least five glomerli in the antennal lobe (Singh & Nayak, 1985). The similarity in fine structure and projection to those of the antennal sensilla strongly suggests that these also have olfactory function.

A number of sensory organs have been identified, on anatomical basis, in the larva (Singh & Singh, 1984), but it remains to be demonstrated which has olfactory function. One of these, the antennal organ, containing 32 dendrites, is a likely candidate for an olfactory organ. Its fine structure is similar to that of the dorsal organ in the house fly larva, which has been suggested to be an olfactory organ on both anatomical and functional grounds (Chu & Axtell, 1971).

Projections from olfactory sensillae into the brain

Axons from the olfactory neurons (about 1200 from each side) project, via the antennal nerve, to the antennal lobe in the deutocerebrum, where they synapse with local interneurons. The dense synaptic connections are seen as spheroidal structures called the glomeruli. The antennal lobe contains at least 22 glomeruli on each side and is thought to be a functional unit involved in the processing of olfactory signals. Different types of sensilla project to different subset of glomeruli. Each sensory axon seems to terminate in only one specific glomerulus (Stocker *et al.*, 1983). Functional mapping, using 2-deoxyglucose labeling method, shows that each class of odorants stimulate neuronal activity in a distinct, although overlapping, subset of glomeruli (Rodrigues & Buchner, 1984; Rodrigues, 1988).

From the antennal glomeruli, higher order projections lead to, via the antennal glomerular tract, among other regions of the brain, the mushroom bodies (or corpora pedunculata) in the protocerebrum. The mushroom bodies are believed to be important in the higher order processing of olfactory information (Heisenberg *et al.*, 1985).

The gustatory system

The taste organ of *Drosophila* is anatomically very similar to that of the much larger blowfly *Phormia*, which has been extensively studied (Dethier, 1976). The taste receptor site is primarily located on the tarsal segment of the leg and on the labellum of the proboscis. 50-67 taste sensilla are located on each half of the labellum in a rather constant pattern: 30-35 A-type bristle with 5 neurons in each, 5-7 B-type bristle with 3 neurons each, and 15-25 sensilla basiconica with 2 neurons in each. One neuron in each bristle is mechanosensory, the others are chemosensory, giving a total of about 145-180 chemosensory neurons (Falk *et al.*, 1976).

MOLECULAR MECHANISM OF OLFACTION & GUSTATION

Olfactory receptors

Although not proven, many studies suggest that specific membrane proteins act as the olfactory receptor molecules in olfactory neurons (Getchell, 1986). Several glycoproteins have been identified uniquely on vertebrate olfactory neurons (Margolis, 1985; Chen & Lancet, 1984; Chen *et al.*, 1986; Allen & Akeson, 1985), but no direct interaction with odorants has been demonstrated. Their functions remain to be established.

Odorant binding proteins

A protein has been isolated from rat based on its high affinity binding to certain odorants (Pevsner *et al.*, 1985). Its localization to the lateral nasal glands and the mucus secreted by these glands suggest a role in concentrating and carrier of odorants (Pevsner *et al.*, 1988a; Pevsner *et al.*, 1988b). A cDNA encoding a similar protein has been cloned from frog (Lee *et al.*, 1987). A sex pheromone binding protein has also been isolated from two species of insects (Vogt & Riddiford, 1981; Gyorgyi *et al.*, 1988), again being soluble and abundant and appears to have carrier function. The insect pheromone binding proteins, however, do not have sequence homology to the vertebrate odorant-binding proteins.

Olfactory signal transduction

An adenylate cyclase, whose activity can be stimulated by a wide variety of odorants, has been identified in the frog and rat olfactory epithelium (Pace *et al.*,

1985; Sklar *et al.*, 1986; Lowe *et al.*, 1989). This odorant stimulation is dependent on GTP, suggesting a link to G proteins. Six GTP-binding protein cDNA species have been cloned from the rat olfactory epithelium, including a G_{olf} which is olfactory neuron-specific (Jones & Reed, 1987, 1989). cAMP is also shown by patch clamping recording to regulate the opening of certain ion channels on olfactory cilia (Nakamura & Gold, 1987). In addition, protein kinase C and a cAMP dependent protein kinase activitity have also been identified in the frog olfactory epithelium (Anholt *et al.*, 1987; Heldman & Lancet, 1988). However, whether the stimulation of adenylate cyclase by odorants is olfactory-specific is in question, since the same stimulation can also be observed in melanophores, pigment cells having no olfactory function (Lerner *et al.*, 1988). In insects, protein kinases have been implicated in the sugar response of the blowfly *Phormia regina* (Amakawa & Ozaki, 1989).

Neurotransmitters in the olfactory system

The dipeptide carnosine (β-alanyl-L-histidine) has been found to be highly concentrated in vertebrate olfactory bulb, and is suggested to be the neurotransmitter in primary olfactory neurons (Margolis, 1974). In *Drosophila*, acetylcholine has been proposed to be a neurotransmitter in the olfactory system, based on heavy [^{3}H]choline uptake in the antennal lobe and antennal glomerular tract (Buchner & Rodrigues, 1983).

Molecular mechanism of taste

Recent studies have begun to identify the molecular components of the gustatory system, and indicate that the four primary taste (sour, salt, sweet, and bitter) may be mediated by diverse mechanisms (Kinnamon, 1988). Apically locally voltage-dependent K^+ channel in vertebrate taste bud cells have been identified and accounts for sour taste transduction (Kinnamon *et al.*, 1988). This K^+ channel could possibly respond directly to changes in H^+ concentration. Evidences also suggest that voltage-independent, amiloride-blockable Na^+ channels on the apical membrane of vertebrate taste cells mediate sodium salt taste transduction (Kinnamon, 1988). Again, specific receptors may not be needed. However, many evidences suggest that sweet taste do involve multiple specific membrane protein receptors Kinnamon, 1988). Adenylate cyclase, cAMP-regulated K^+ channels, and Na^+ channels have also been linked to sweet taste transduction. Specific receptors and Ca^{++} mediated second-messenger system have been suggested for the bitter taste transduction (Akabas *et al.*, 1988).

In *Drosophila*, three sugar receptor sites (pyranose, furanose, trehalose) have been suggested based on discrimination by protease (Tanimura & Shimada, 1981) and by mutations (Siddiqi & Rodrigues, 1980, Tanimura *et al.*, 1982; Isono & Kikuchi,

1974). Independent receptor sites for Na^+ and K^+ have similarly been suggested (Siddiqi, personal communication). A K^+ channel mutant, *Shaker*, exhibit greatly reduced response to sucrose and increased acceptance of NaCl (Rodrigues, personal communication), suggesting that K^+ channels are involved in the sugar and salt response.

MEASUREMENT OF *DROSOPHILA* CHEMOSENSORY RESPONSES

Both larvae and adults respond to a large number of volatile chemicals with stereotyped behavior. The adults migrate toward attractants, away from repellents, and exhibit complex courtship behaviors in response to sex pheromones. The larvae are, however, attracted by all odorants they can sense, even those that are repellent to the adults. Since some genes seem to be used in both olfactory systems, the difference presumably lies at the contral steps of olfactory information processing.

The olfactory response can be measured by both behavioral and electrophysiological methods. Most behavioral assays (Kikuchi, 1973; Fuyama, 1976; Rodrigues & Siddiqi, 1978; Rodrigues, 1980; Monte *et al.*, 1989) let flies, either adults or larvae, choose between two environments, one of which contains the test odorant. The relative distribution of flies is used as an indicator of their preference to that odorant. This type of assay can be modified to trap the flies to one side, thereby enhancing the response to an attractant (Woodard *et al.*, 1989). We have designed a large trap assay, in which flies are trapped in milk bottles containing odorant. The response, as measured by the percentage of flies trapped, is dependent on the odorant stimulation (Table 1A). The response is apparently perceived through the antennae, as surgically removing both antennae abolishes the response to food but not the response to light (Table 1A, 1B). A different type of assay utilizes the observation that when flies are exposed to a short pulse of strong odorant stimulation, a jump response is elicited (Mike McKenna, personal communication). Behaviors in all of these assays are dose dependent and chemical specific.

The electroantennogram (EAG) has been developed to record from the antennal surface the electrical response to odorants (Siddiqi, 1983; Venard & Pichon, 1984). Extracellular recordings from a single sensillum can also be made, and are able to detect the activity of a single neuron: most neurons respond to several classes of odorants, although at different threshold and with different intensity, while some respond uniquely to one class of chemicals (Siddiqi & Rodrigues, 1980).

Whether two chemicals are perceived through a common pathway has been examined experimentally by measuring the response to one chemical in a uniform background of the second chemical (Rodrigues, 1980). Chemicals that do not

Table 1
Large trap olfactory assay

	Flies		Stimulus	% Inside trap (±SEM)
(A)	CS-5		food	92 ± 3 (n=8)
	CS-5		agar	16 ± 6 (n=8)
	CS-5	ant$^-$	food	25 ± 9 (n=5)
(B)	CS-5		light	21
	CS-5		dark	2
	CS-5	ant$^-$	light	25
	CS-5	ant$^-$	dark	0
(C)	CS-5		food	74
	CS-5	ant$^-$	food	8

About 100-150 flies are put in a 30×30×30 cm plastic cage, with two sheets of water-saturated Whatman #1 filter to maintain humidity. In the cage is also placed a 250 ml standard milk bottle with a paper funnel over the mouth. The tip of the funnel is 0.2 cm in diameter. 30 ml of stimulant, standard fly food or plain 1% agar, is placed in the bottle. Flies are allowed usually 24 hr in dark to enter the trap. CS-5 is derived from the Canton-Special strain with an isogenized X chromosome (Monte *et al.*, 1989) and is the parent stock for the EMS mutagenesis.

(A) Flies are attracted strongly by food but not by agar. Surgically deantennated flies do not respond to food odor. (B) Light can also be used as a stimulus to attract flies into the trap. Deantennated flies can enter the trap in response to light, therefore is not affected in their orientation and general neural functions. (C) Flies behave independently in a mixing experiment where normal and deantennated CS-5 flies are mixed and tested in the same trap assay. This is a simulation of the mutant-screening situation, where rare mutants are tested with a large number of wild-type flies.

block the response to each other are interpreted as being perceived through independent pathways. Pairwise tests with 18 chemicals have suggested the existence of at least five pathways, corresponding to five chemical groups: acid, acetate ester, alcohol, aldehyde, and ketone. Analogous experiments can be performed with EAG: if the response to two chemicals simultaneously applied to the antenna is additive, the two chemicals are interpreted as being preceived through different pathways (Borst, 1984). Tests with 40 chemicals reflect the existence of 8 different receptor classes (Rodrigues, 1980; Siddiqi, 1983). It is expected that the number of pathways will increase as more chemicals are tested.

The taste response of the blowfly *Phormia* has been extensively characterized both behaviorally and electrophysiologically (Dethier, 1976). The same assays can also be applied to *Drosophila*, yielding similar results. Stimulation of a single sensory hair in the labellum or the tarsal segments of the legs with sucrose solution will cause an extension of the proboscis (Tompkins *et al.*, 1979; Tompkins & Barnhart, 1982). The fly is about equally responsive to maltose, glucose and fructose, and much less responsive to galactose, lactose and xylose (Siddiqi & Rodrigues, 1980). Quinine and NaCl inhibits the extension of proboscis.

Ingestion of sugar solution can also be visualized by adding dye in the solution and examine whether the fly's gut has been stained by the dye (Falk &

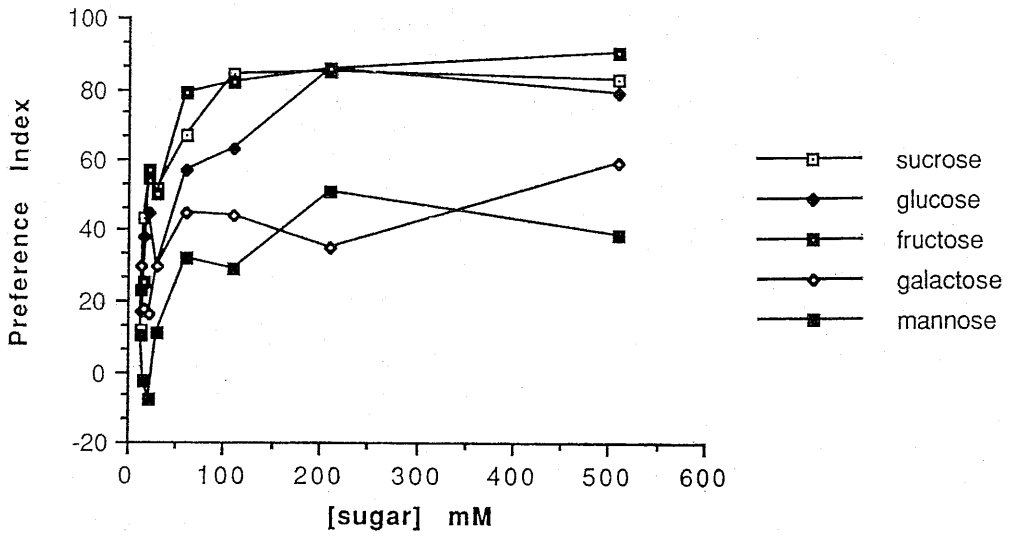

Fig. 1. Response to sugars in the dye-ingestion gustatory assay. 24-well assay plate is cut from a 96-well microtiter plate. Each well is filled with 0.7% agar containing alternately 0.05% blue food dye or 0.2% red food dye. These dye concentrations are experimentally chosen for easy visualization and very low interference with the assay: flies do not show preference for either color. Sugars are added at the indicated concentration to alternate wells. Half of the assays in each experiment has sugar in the red well, the other half of the assays has sugar in the blue well. Again, no significant interference from the dyes has been observed. 10-100 flies are first starved in a vial containing a moist sponge for 5 hours, then put into a 9×6×4 cm plastic box containing the assay plate. The flies are allowed to drink for 30 min in the dark. They are then anesthesized and observed under microscope to score whether their gut contain red, blue or both dye, as an indication of which well they had ingested from. The Preference Index is defined as PI=×100 (S−C)/T where S is the number of flies that drank from the stimulus side, C is the number of flies that drank from the control blank side, T is the total number of flies in the assay.

Atidia, 1975; Tanimura *et al.*, 1982). Sucrose, glucose and fructose elicit strong response with similar dose dependence, while galactose and mannose elicit a weaker stimulatory response (Fig. 1). NaCl and KCl at high concentrations both inhibit the ingestion response to sucrose solution. However, NaCl at concentrations lower than 100 mM actually stimulates ingestion (Fig. 2; also Arora *et al.*, 1987). We have also developed a plate assay testing the taste preference of larvae as indicated by their relative distribution (Sun, unpublished results). Behaviors of larvae are similar to those of adults.

Electrophysiological recordings have shown that of the four sensory neurons in each bristle, one is responsible for sugar preception (S cell), one for water (W cell, inhibited by salts and sugars), two for salt (L1 and L2 cell) (Fujishiro *et al.*, 1984). Quinine inhibits the response of all four, although at different level.

It is essential to have reliable and sensitive assays to measure the fly's

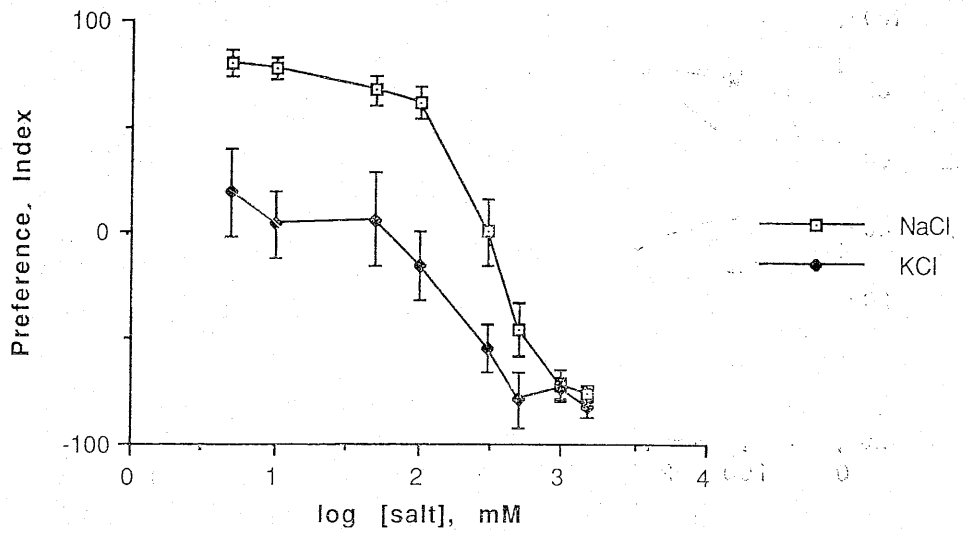

Fig. 2. Taste response to salts vs. 100 mM sucrose. The dye-ingestion assay as described in Fig. 1 is performed with 100 mM sucrose in all wells and NaCl or KCl at the indicated concentration, as the stimulant, in alternate wells. Negative values of PI indicate that the salt can suppress the ingestion response to sucrose.

response to chemosensory stimulation. The assays are needed for screening and phenotypically characterizing mutants. It is important to have multiple assays for both adult and larva. An ideal assay should meet the following criteria: low variability; with strong response to stimulus, and low background response; response is quantiatively dependent on stimulus concentration; respond to multiple odorants; simple to perform; inexpensive; can scale up for mass screening; individuals behave independently; individuals can be repeatedly tested. We have tried many possibilities. Although none meet all the criteria, several assays are satisfactory. Better assays are continuously being sought.

GENETIC ANALYSIS

olf and *gust* mutants

Mutations affecting the olfactory response have been isolated (Kikuchi, 1973; Rodrigues & Siddiqi, 1978; Rodrigus, 1980; Siddiqi, 1983; Aceves-Pina & Quinn, 1979). Rodrigues and Siddiqi have isolated several mutants on the X chromosome at very high frequency. At least 7 complementation groups have now been identified and mapped. Four of these, *olfA*, *olfB*, *olfC* and *olfD*, have multiple alleles. *olfA*, *olfE* and *olfC* map to a small region, near the *singed* (*sn*) locus of the X chromosome, as if they were part of a clustered multigene family. Carlson's lab at Yale has also isolated several mutants from the X chromosome, using several assays (Woodard *et al.*, 1989; Helfand & Carlson, 1989; Carlson, personal

communication). A mutation has also been found on the second chromosome (Kikuchi, 1973).

All existing *olf* mutations are recessive. In all mutants, both adult and larva exhibit the phenotype (Siddiqi, 1983). Some mutants are specific in not responding to a single class of odorant. The *olfC* mutant does not respond to the strong attractant ethyl acetate and other acetate esters, but is, in general, normal in response, both behaviorally and electrophysiologically, to other groups of chemicals (Siddiqi, 1983; Venard & Pichon, 1984). This suggests that the defect in *olfC* is at a peripheral stage of olfaction, possibly in a receptor. Some mutants are defective toward two classes of chemicals. Two mutations, *olfD* and *smellblind* (*sbl*), allelic to each other, abolish the responses to all chemicals tested and also affect taste response (Carlson, personal communication). Several mutants were found also to have visual defect, suggesting certain components are shared by the olfactory and visual system (Woodard *et al.*, 1989; Carlson, personal communication).

Specific mutants have been isolated for NaCl, pyranose, and quinine (Rodrigues & Siddiqi, 1978; Tompkins *et al.*, 1979; Falk & Atida, 1975). Also identified were mutants defective for all three types of chemicals or various combinations of them (Rodrigues & Siddiqi, 1978; Tompkins *et al.*, 1979). In sensillum recordings, several mutants respond normally to sugars, salts, and quinine, and presumably their defect is at a central processing step (Siddiqi & Rodrigues, 1980). *gustA*, which does not respond to pyranoses behaviorally, also does not respond in sensillum recordings, indicating that its defect is peripheral, possibly at the receptor. One mutation, *gust B*, makes the S cell excited by salts, hence the mutant fly is attracted to NaCl at concentrations repellent to wild type flies (Arora *et al.*, 1987).

Mutagenesis and mutant screening

Conditions for the screening are experimentally chosen to induce a strong response (about 90%) in the wild type flies. We have shown that behavior of a fly in the large trap assay is independent of other flies by testing a mixed population of surgically deantennated flies (presumably having no olfactory function) and wild type flies (Table 1C). This is important in that the rare mutants would not be influenced by wild type flies, and hence be obscured in the screening.

A population of 4000 EMS-mutagenized (following the protocol of Lewis & Bacher, 1968) F1 male flies are put through the large trap assay. Flies that failed the assays (i.e., did not enter the trap) are collected, retested and rested. Again, flies that failed the second test are collected. This process enriches the proportion of mutants in the population. After the enrichments, individual males from the enriched population are mated individually to attached-X virgin females to

Table 2
Preliminary characterizations of putative olfactory mutants

(A) large trap assay

Flies	Stimulus	% Inside Trap (± SEM)
control males	food	87 ± 3 (n=12)
control females	food	89 ± 2 (n=13)
4-3 male	food	44
4-3 female	food	85

(B) small trap assay

Flies	% Inside Trap (±SEM)	% of low response
CS-5	81 ± 5 (n= 8)	0
4-3	36 ± 5 (n=15)	87
	34 ± 12 (n=10)	70
GH-3	8 ± 3 (n= 9)	100
	19 ± 5 (n=14)	100

EMS-mutagenized F1 males are mass screened with the large trap assay for 2-3 cycles. Flies that failed the assays were individually mated to attached-X virgin females to establish lines. In such line, all males receive the paternal mutagenized X chromosome, while all females receive the maternal non-mutagenized attached-X chromosome. (A) Males of mutant line 4-3 exhibits a lower response to food odor in the large trap assay, while females of the same line respond well. Both male and female flies of other lines show normal response. (B) Mutant 4-3 and GH3 both show low responses to food in the small trap assay. The low response is even more striking when one compares the percentage of assays with a response of lower than 60% inside the trap.

establish lines. These lines are then tested with the small trap assay for defective chemosensory response. Two putative mutants, 4-3 and GH3 (Table 2) are thus isolated. Flies of these two mutant lines respond poorly to fly food as stimulus in the small trap assay. Mutant 4-3 also exhibits significantly lower response to food odor in the large trap assay. This defect is, as expected, due to an X-linked mutation, as evidenced by the normal response of the females, which carry the unmutagenized attached-X chromosomes, in the 4-3 line. Mutant 4-3 has been tested for its ability to respond to light in the large trap assay, and is apparently normal in this respect (data not shown). Mutant GH3 is also defective in responding to ethanol (data not shown). Further characterizations are being performed.

In addition to the traditional mutagenesis method using chemical mutagens or X-ray irradition, transposable elements can also be used as a way of mutagenesis. P element is a class of transposable element present in multiple copies in the genome of certain *Drosophila melanogaster* strains (P strains), but absent in others (M strains). Their transpositions are normally repressed in the P strains. When males of a P strain are mated with females of an M strain, the P elements are relieved from the repression and transpose at high frequency, leading to high rates of mutation, chromosome rearrangement, male recombination and reduced

fertility, a phenomenon called hybrid dysgenesis (Kidwell & Kidwell, 1977; Engels, 1983). The frequency at which mutations are induced by hybrid dysgenesis varies at different loci, ranging from 9×10^{-5} to 8×10^{-4} (Green, 1977). P element-mediated hybrid dysgenesis has been used successfully as a method of transposon-tagging (Searles *et al.*, 1983), and provides a convenient way for cloning a genetic locus when the mutant phenotype can be easily scored for. A P element inserted in the locus allows the region to be identified and isolated by hybridization with a cloned P element.

We are using a new scheme (Robertson *et al.*, 1988) that uses a single engineered P element (Δ2-3) which provides very high transposase activity but itself cannot be transposed. When this P element is brought together with another chromosome (the second chromosome from the Birmingham strain) bearing 17 nonautonomous P elements (cannot produce functional transposase, therefore can be transposed only with the help of transposase provided by others), high mutation frequency can be induced. The two chromosomes can then be segregated, so the P insertions will immediately become stabilized. This prevents the problem of instability of P insertional mutations. The mating scheme is as described (Robertson *et al.*, 1988). F2 males are screened for X-linked mutations. Several putative mutant have been isolated and are being characterized (data not shown).

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