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acj6: A gene affecting olfactory physiology and behavior in *Drosophila*

(antennae/chemosensation/signal transduction/brain/sensory system)

RICHARD K. AYER, JR., AND JOHN CARLSON

Department of Biology, Yale University, New Haven, CT 06511

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ABSTRACT **Mutations affecting olfactory behavior provide material for use in molecular studies of olfaction in *Drosophila melanogaster*. Using the electroantennogram (EAG), a measure of antennal physiology, we have found an adult antennal defect in the olfactory behavioral mutant abnormal chemosensory jump 6 (*acj6*). The *acj6* EAG defect was mapped to a single locus and the same mutation was found to be responsible for both reduction in EAG amplitude and diminished behavioral response, as if reduced antennal responsiveness to odorant is responsible for abnormal chemosensory behavior in the mutant. *acj6* larval olfactory behavior is also abnormal; the mutation seems to alter cellular processes necessary for olfaction at both developmental stages. The *acj6* mutation exhibits specificity in that visual system function appears normal in larvae and adults. These experiments provide evidence that the *acj6* gene encodes a product required for olfactory signal transduction.**

The use of sensitive electrophysiological measures to study olfaction in a genetically manipulable animal is potentially fruitful but largely unexplored. Physiological techniques have proven invaluable in characterization of *Drosophila melanogaster* mutants with defective ion channels [e.g., Shaker (1–4) and para (5, 6)], visual receptor molecules [e.g., *ninaE* (7–9)], and other signal transduction molecules [e.g., *norpA* (10, 11)]. Electrophysiological analysis has proven useful in determining the sites of action of mutations that were initially isolated in behavioral screens [*Sh* (12), *para* (13), and *norpA* (14)]. Demonstration of a physiological phenotype associated with a mutant behavioral phenotype can further aid in characterizing such mutations at genetic [*norpA* (15, 16)] and molecular [*ninaE* (17)] levels.

Abnormal behavior of *Drosophila* olfactory mutants (18–22) can be due to a wide range of defects in processes from odorant sensation to directed movement. Before extensive genetic and molecular work on a mutant locus is undertaken it may be useful to have a basic understanding of where the gene product is used in the olfactory pathway. Mutations affecting olfactory receptors and other signal transduction molecules are expected to disrupt olfaction in peripheral sensory neurons; in adult *Drosophila*, many of these cells are localized in the antenna (23), the principal olfactory organ (24). Using the electroantennogram (EAG; refs. 25–27), an extracellular measure of the potential change produced in the antenna by olfactory stimulation, it may be possible to determine whether an olfactory mutation alters behavior by disrupting reception or signal transduction, as opposed to affecting central processing or motor activity. The *Drosophila* antenna contains first-order neurons only; these cells make their first synapse in the brain (28), some distance away. This anatomical separation implies that a mutation affecting the EAG is likely to act in peripheral sensory neurons or possibly in nonneuronal support cells, but it is unlikely to act only in postsynaptic neurons in the central nervous system.

In addition to characterizing an olfactory mutant with the EAG, information about the affected gene may also be gained by analyzing the mutant's olfactory function at different stages of development. *Drosophila* larvae have a sensitive olfactory system whose function can be measured by behavioral assays (29–31). The larval olfactory organs have different developmental origins and morphology from their adult counterparts, and are histolyzed during metamorphosis (32). Thus, while genes required for olfactory transduction might be used in both adult and larval organs, genes that play roles in nonneuronal support cells unique to the adult antenna might not have larval olfactory phenotypes.

The *Drosophila* abnormal chemosensory jump (*acj*) mutants were isolated on the basis of decreased olfactory jump response, an adult behavior elicited by exposure to ethyl acetate vapor (20). In addition to mutations that alter an odorant-elicited jump response by disrupting conduction of nervous impulses, central nervous system processing of sensory information, or specific muscle movements, some fraction of *acj* mutations should alter the jump response by disrupting odorant detection. As with other olfactory behaviors (19, 21, 24), the jump response has been shown to be mediated primarily by the antenna (20). The EAG was therefore used to determine whether any of the *acj* mutations affected antennal odorant detection (20).

Here we show that the *Drosophila* olfactory mutant *acj6* has sharply reduced EAG amplitude, a parameter dependent on odorant dose and highly reproducible for wild-type animals. Furthermore, we genetically characterize *acj6* based on its decreased EAG amplitude and provide evidence that this physiological lesion is responsible for the observed defect in olfactory behavior. We show that *acj6* larvae also have defective olfactory behavior, placing additional constraints on the nature of the mutant locus. Moreover, we provide evidence that the *acj6* visual system is normal in both adult physiological tests and larval behavioral tests, suggesting a degree of specificity to the *acj6* defect.

MATERIALS AND METHODS

Fly Stocks and Cultures. Canton-S-5 (CS-5) is derived from a Canton-S wild-type strain; its origin, and that of *acj6*, an X chromosome-linked, ethyl methanesulfonate-generated mutant, are described elsewhere (20). The *acj6* X chromosome was maintained over the *C(1)A y*-attached X chromosome; only the male flies expressed the X-linked mutation. To compensate for possible genetic background effects, the CS-5 X chromosome, the parental wild-type control in these ex-

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Abbreviations: EAG, electroantennogram; ERG, electroretinogram; CS-5, Canton-S-5.

periments, was also kept over *C(1)A y*. *Drosophila* were cultured as described by Monte *et al.* (31).

Electrophysiological Recording. EAGs were measured from male flies 1–5 days after eclosion. Each fly was held during recording in a truncated 200-μl plastic pipette tip, trimmed such that the anterior aspect of the fly's head and antennae were exposed. Recording and ground electrodes were unbroken glass micropipettes filled with *Drosophila* Ringer's (33), connected to a high-impedance DC amplifier whose output was viewed on an oscilloscope. With the ground electrode inserted into the head capsule, the recording electrode was brought into electrical contact with the posterior surface of the third antennal segment near the base of the arista. A constant air stream of ≈1 liter/min was directed at the fly. Pulses of odor, produced by passing 3 ml of air from a syringe over a filter disk saturated with odorant diluted in water, were introduced into the constant air stream (34). Measurable EAG deflections were recorded when the filter disk was left dry, possibly indicating antennal detection of mechanical changes in the air stream; for purposes of this study, such a stimulus is referred to as "mechanical." Chemicals used as odorant sources for EAG recording and for the behavioral assays outlined below were of the highest purity available from Fluka.

Electroretinogram (ERG) recordings were performed as described elsewhere (10), except that flies were immobilized during recording in the same manner as for the EAG (see above). Light stimuli were provided by a fiber-optic microscope illuminator, focused on the fly, and initiated and terminated via a mechanical shutter interposed in the light path. The level of illumination of the halogen light source was 10,000 lx, measured at the plane of the fly.

Adult Behavioral Assays. Olfactory jump response to undiluted ethyl acetate was assayed as outlined by McKenna *et al.* (20). Briefly, a single fly was inserted into a tissue culture tube through which room air was being drawn. After the fly locomoted vertically up the side wall of the tube, it was given a pulse of odorized air; a jump was scored if the fly reached the bottom of the tube within 3 sec. By convention, inactive flies that did not climb up the tube were excluded from the jump assay. Flies were tested in groups of 30; values in Table 1 indicate mean percentage (±SEM) of flies in each group that jumped.

A nonclimbing index, derived in conjunction with the jump assay, indicates the percentage of flies that failed to locomote up the wall of the jump apparatus.

Larval Behavioral Assays. Larval olfaction was assayed as outlined by Monte *et al.* (31), modified to accommodate the fact that the *acj6* chromosome was maintained in a stock in which only males expressed the mutant phenotype (see above). Briefly, 150–200 larvae were placed in the center of an agarose Petri plate containing two diametrically opposed filter discs; one contained 25 μl of odorant, and the other contained a diluent control. After 5 min, larvae on the stimulus (S) and the control (C) halves of the plate were transferred to separate culture vials. After the adult flies eclosed, males in S and C vials were counted; a male response index (RI) is calculated as $RI = (S - C)/(S + C)$.

The larval phototaxis assay was as outlined by Lilly and Carlson (22), again with modification as described above. A Petri plate sectioned into quadrants contained alternating dark (3% food dye) and clear agarose. The dish was placed on a light box in a dark room and larvae, placed originally at the center of the dish, partitioned preferentially onto the dark quadrants. After 5 min, larvae on dark (D) or clear (C) quadrants were transferred to separate vials. Eclosing adult males were counted and an index, $RI = (D - C)/(D + C)$, was determined. A dose–response curve was generated by adding increasing percentages of dye to the clear quadrants. Since dye concentration in the dark quadrants remained fixed, the light intensity differential between the two pairs of quadrants decreased when increasing dye concentrations were added to the clear quadrants.

Meiotic Recombination Mapping of the *acj6* EAG Phenotype. Female flies, homozygous for the markers yellow (map position 0.0), crossveinless (map position 13.7), vermilion (map position 33.0), and forked (map position 56.7), were crossed to *acj6* males, and their female offspring were crossed to males carrying a dominantly marked X chromosome (FM7c). Under CO_2 anesthesia, F_2 male offspring were separated into the eight classes indicated in Fig. 4. Two days later, the EAG of animals from each recombinant class was recorded. Recombination mapping using the marked chromosome vermilion (map position 33.0), miniature (map position 36.1), garnet (map position 44.4), scalloped (map position 51.5), and forked (map position 56.7) was accomplished by the same methodology.

RESULTS AND DISCUSSION

Fig. 1*A* shows that *acj6* has an abnormal EAG: The response is greatly reduced for the mutant. Response magnitude was quantified by recording EAG amplitude, defined as the peak odorant-stimulated voltage change. Average *acj6* EAG amplitude is decreased at all tested concentrations of the odorants ethyl acetate (Fig. 1*B*) and benzaldehyde (Fig. 1*C*). The reduction ranges from 70% at the lowest doses to 25% at the highest concentrations of both odorants. These results are consistent with behavioral data which showed that jump response was reduced to benzaldehyde as well as to ethyl acetate (20).

The diminished EAG amplitude of *acj6* at a given odorant concentration may reflect diminished antennal sensory input to the *acj6* central nervous system during chemosensory stimulation. Wild-type odorant jump response, like EAG amplitude, decreases with decreasing stimulus concentration (19, 20). Thus, the reduced chemosensory jump response in *acj6* adults may be a function of a reduced antennal odorant response. Light microscopy of the antennal surface (23, 35) reveals no obvious defects in gross morphology or in the pattern of olfactory sensilla to account for reduced EAG responses or abnormal olfactory behavior.

The *acj6* physiological defect has been demonstrated for two different odorants. The EAG response to water vapor is reduced as well, possibly representing a defect in hygroreception, a sensory function known to be mediated by the antennae of some insects (26), including *Drosophila* (24). Abnormal peripheral signaling, however, does not extend to include the visual system. Limited testing previously showed *acj6* to have a qualitatively normal light-evoked ERG (20). Fig. 2 shows the results of quantitative testing of the *acj6* visual system in which ERG amplitude is found not to be significantly different from wild type. Specifically, the amplitudes of three ERG parameters, the on and off transients and the receptor potential, were measured for wild-type and *acj6* flies. Normal receptor potential amplitude in the mutant suggests normal functioning of the photoreceptor cells in the *acj6* retina, while the presence of on and off transients suggests normal function in the second-order neurons of the visual system (36).

Drosophila larvae also respond to olfactory stimuli (29–31). While the developmental origins of the larval olfactory organ are distinct from those of the adult antenna (32), both could conceivably share transduction pathway components. Fig. 3*A* shows that *acj6* larvae are abnormal in olfactory behavior. The dose–response curves indicate that the mutant is essentially unresponsive at some concentrations of ethyl acetate that are strongly attractive to wild type. In addition to the lower response documented in the figure, some 20–60% of *acj6* larvae were found writhing in a clump in the center of the plate at the end of the assay; by convention (31),

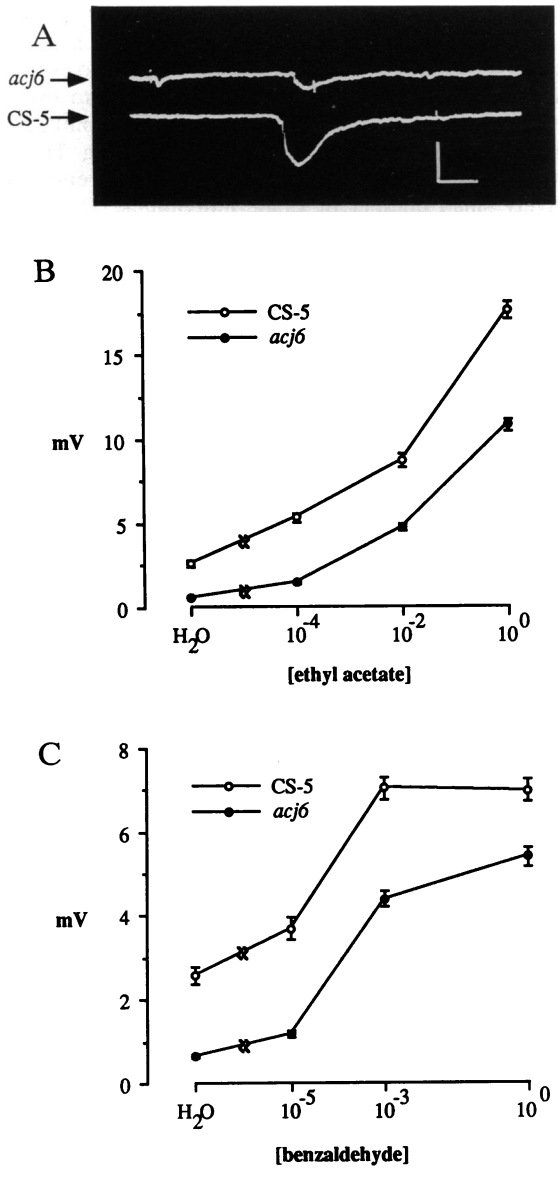

FIG. 1. Reduced EAG amplitude of the olfactory mutant *acj6*. (*A*) Typical EAG responses to a 10^{-4} dilution of ethyl acetate for *acj6* (upper trace) and the parental wild-type strain CS-5 (lower trace). At this odorant concentration, the amplitude of the *acj6* response is ≈25% of wild type. [Scale bar = 5 mV (ordinate) and 500 msec (abscissa).] (*B* and *C*) Dose–response plots of the peak EAG amplitude versus the log of the odorant concentration for both wild-type (CS-5) and mutant (*acj6*) flies for ethyl acetate (*B*) and benzaldehyde (*C*). Error bars, too small to be seen in some cases, indicate SEM; $32 \leq n \leq 40$; differences between means are significant at $P < 0.05$ for all concentrations of both odorants. Both chemicals were diluted in water.

they were excluded from olfactory response calculation. This clumping appears to represent a new phenotype, in that wild-type larvae show much less clumping, even at very low odorant concentrations. At higher odorant doses, *acj6* response is similar to wild type; the mutant larvae disperse and move toward the odorant disk. In contrast, *acj6* larval light avoidance (22) is normal over a wide range of stimulus levels (Fig. 3*B*). Thus, in a behavior requiring transduction, central processing, and motor response to a visual stimulus, mutant performance is indistinguishable from wild type.

In addition to EAG and olfactory behavior phenotypes, *acj6* flies move less than wild type in response to agitation of their culture vials. Moreover, while wild-type flies quickly

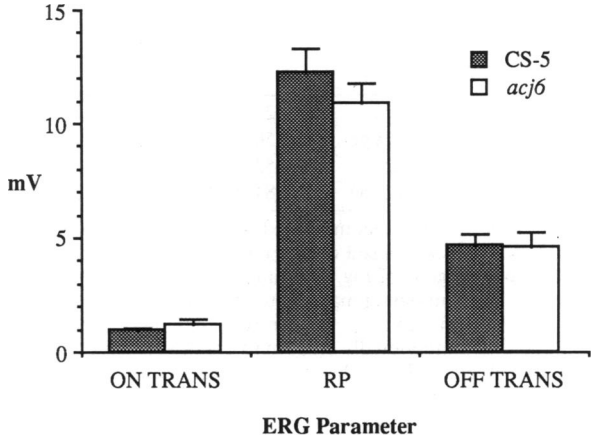

FIG. 2. Normal ERG amplitude of *acj6*. The amplitudes of the receptor potential (RP), the on transient (ON TRANS), and the off transient (OFF TRANS) were measured for wild-type and *acj6* flies. The amplitude of the receptor potential was measured at the end of the 500-msec light pulse stimulus. Each value represents the average of 10 flies (±SEM).

crawl up the wall after being placed in the jump apparatus, ≈10% of *acj6* flies do not and are excluded from the assay (see nonclimbing index; Table 1). This nonclimbing fraction can move; tapping the jump apparatus increases their activity. The reduced climbing frequency, like the reduced sensitivity to agitation of vials, could reflect a reduced responsiveness to disturbance. These two phenotypes may be

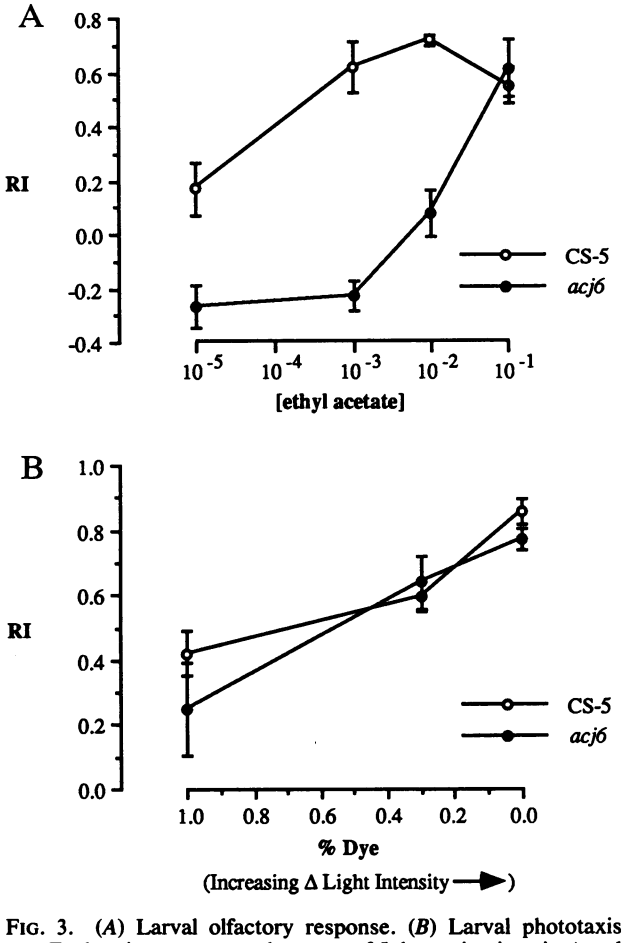

FIG. 3. (*A*) Larval olfactory response. (*B*) Larval phototaxis assay. Each point represents the mean of 5 determinations in *A* and 7–12 determinations in *B*; error bars indicate SEM. In *B*, the abscissa indicates the percentage of dye added to the clear quadrants.

Table 1. Cosegregation of *acj6* phenotypes

Recombinant class	EAG amplitude	Olfactory jump response	Nonclimbing index
y cv v +	Mutant	27 ± 3 (15)	10 ± 2 (15)
	Wild type	56 ± 3 (10)	1 ± 1 (10)
+ + + f	Mutant	52 ± 4 (15)	18 ± 2 (15)
	Wild type	84 ± 2 (10)	2 ± 1 (10)

+ + + f or *y cv v +* recombinant flies were judged to be mutant or wild type for EAG based on the bimodal amplitude distribution described in the legend of Fig. 4. From these flies, three *y cv v +* and three *+ + + f* recombinant males with mutant EAG phenotypes and two *y cv v +* and two *+ + + f* recombinants with wild-type phenotypes were individually crossed to *C(1)A y* females to establish 10 different lines; males within each line carried the same X chromosome as their father. The lines were then scored for olfactory jump behavior and climbing phenotypes. Five groups of 30 flies were tested for each line; values in parentheses indicate the total number of groups tested for each recombinant class. As markers on the *y cv v f* chromosome may also affect the jump response, comparisons were only made between mutant and wild-type lines carrying identical markers. For recombinant lines within either *y cv v +* or *+ + + f* classes, average jump scores for lines with mutant EAG phenotypes were uniformly lower than the scores for lines with wild-type EAG phenotypes. The indicated differences between *acj6*$^-$ flies and their *acj6*$^+$ counterparts are statistically significant at $P < 0.05$.

caused in part by reduced sensory input from mechanical stimuli, which are also sensed in part through the antenna. In fact, the mutant has reduced EAG amplitudes in response to mechanical stimulation (data not shown; see *Materials and Methods* for definition).

It is important to note that the decrease in EAG response is not due exclusively to a defect in response to water vapor or mechanical stimulation. Fig. 1 shows that the *acj6* EAG is abnormal even when water is absent (i.e., at undiluted concentrations). Inspection of the response curve to ethyl acetate reveals that the difference (<2 mV) between the mean responses of mutant and wild type to water vapor is less than the differences (3.8, 4.0, and 6.9 mV, respectively) between the responses to increasing doses of ethyl acetate, which are delivered in the same manner as for water vapor, with equivalent mechanical stimulation. The simplest explanation of these results is that *acj6* has a reduced response to ethyl acetate vapor. Defective response to ethyl acetate vapor has been confirmed in experiments using a more sophisticated odorant delivery system (37) in which ethyl acetate is diluted in paraffin oil and the vapor is delivered with negligible mechanical stimulation (E. Alcorta, personal communication). The dose dependence of the defect in response to benzaldehyde is less clear, and response to benzaldehyde has not been examined with other delivery methods.

The data indicate that *acj6* affects olfaction at each of two distinct developmental stages. The finding that EAG is abnormal in the adult fly suggests that the behavioral defect may be a function of abnormal signal transduction; we note that the EAG is believed to measure primarily the summed receptor potentials of the antennal neurons, as opposed to their action potentials (38). Normal visual response at both stages suggests there is at least some specificity to the *acj6* defect. Using only physiological techniques, it would be difficult to determine exactly how *acj6* affects olfaction. In contrast, physiological examination of the mutant phenotype, combined with established methods for genetic and molecular characterization of genes in *Drosophila* should lead to a better understanding of how the mutation disrupts normal olfactory function. Toward this end, we have begun a genetic analysis of *acj6*. Using recombination mapping, we have localized the mutation responsible for the EAG defect.

Existence of a physiological phenotype has greatly facilitated recombination mapping of *acj6*. Mapping based on behavior alone generally requires the testing of populations of identical recombinant flies, or large numbers of single individuals if a single fly assay is available, in order to determine the absence or presence of the mutation. Due to the large difference between wild-type and *acj6* EAG amplitudes and the reproducibility of the measurements (see Fig. 1), a map position can readily be ascertained by measurement of a relatively small number of single individuals. The reduced EAG amplitude of *acj6* was recombinationally mapped using the marked X chromosome yellow, crossveinless, vermilion, forked (*y cv v f*). Fig. 4 shows that the mutation responsible for the defect lies between *v* and *f*, approximately at map position 51. A second recombination mapping experiment, using the markers vermilion, miniature, garnet, scalloped, and forked (*v m g sd f*) confirmed our localization between *v* and *f* and allowed us to map the mutation at higher resolution to the *g–sd* interval. On the basis of 111 recombinants in the *g–sd* interval, we assign the EAG defect to map position 49.4.

Through additional genetic analysis, it has been determined that the *acj6* olfactory behavior and EAG abnormalities are likely to be caused by a single mutation on the X chromosome. Table 1 shows the chemosensory jump scores of recombinant flies and illustrates that decreased olfactory jumping cosegregates with reduced EAG amplitude. Reduced climbing also cosegregates with the olfactory defects, as if all three *acj6* phenotypes are caused by the same mutational event. Limited data (not shown) suggest that the deficit in larval olfactory behavior also cosegregates with the adult phenotypes. Mosaic mapping of the foci of the EAG and olfactory jump phenotypes might clarify further the relationship between these defects.

These studies provide evidence that the *acj6* gene product is required for olfactory signal transduction. We have used the EAG to demonstrate and genetically map a physiological defect in the *acj6* olfactory behavior mutant. Furthermore,

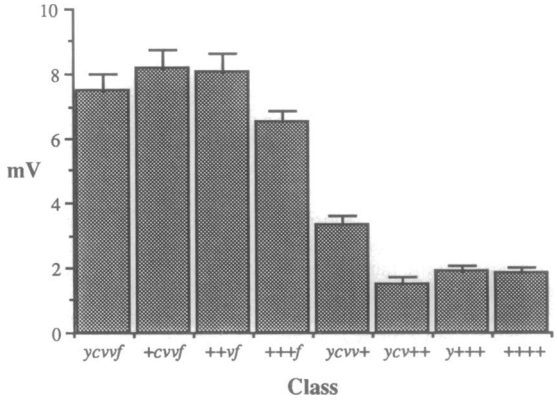

FIG. 4. Recombinational mapping of the *acj6* EAG phenotype using the multiply marked X chromosome yellow, crossveinless, vermilion, forked (*y cv v f*). The two parental X-chromosome classes, *y cv v f* and *+ + + +* (carrying *acj6*) have clearly different amplitudes. Flies representing the recombinant classes *+ cv v f*, *+ + v f*, *y + + +*, and *y cv + +* uniformly had EAG amplitudes characteristic of one of the two parental classes. Individual flies of the *+ + + f* and *y cv v +* classes had either mutant or wild-type EAG amplitudes. Since the distribution of amplitudes within these two classes was clearly bimodal, it was possible to individually score the EAG phenotype of each recombinant within the *v–f* interval, placing *acj6* approximately at genetic position 51 on the X chromosome. The amplitude data in the figure are for application of ethyl acetate at a 10^{-4} dilution; consistent mapping results were obtained when the recombinant flies were tested over the range of concentrations of ethyl acetate and benzaldehyde used in Fig. 1 ($8 \leq n \leq 10$; error bars indicate SEM). Additional flies in the *+ + + f* ($n = 55$) and *y cv v +* ($n = 50$) classes were subsequently tested to localize the mutation at higher resolution.

we have shown that a single mutational event is likely responsible for altering both olfactory behavior and antennal physiology of *acj6* adult flies. *Drosophila* mutants with defective olfactory behavior (18–22) or abnormal EAG (37, 39) have been described; however, in no previous case has an EAG defect been shown to arise from mutation of a single gene or to cosegregate with a behavioral defect. In addition, we have demonstrated a larval olfactory behavior deficit associated with the *acj6* chromosome. These results provide evidence that the *acj6* gene product has a function common to both adult and larval olfactory systems; we do not know whether it also functions in other systems of the fly. Further genetic analysis, including the isolation of additional alleles, and the eventual molecular characterization of *acj6* may clarify the requirement of this gene for olfactory transduction in *Drosophila* and may provide clearer understanding of olfaction in other organisms.

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