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The *east* Gene of *Drosophila melanogaster* Is Expressed in the Developing Embryonic Nervous System and Is Required for Normal Olfactory and Gustatory Responses of the Adult

K. VIJAYRAGHAVAN,*[1] JAGDEEP KAUR, JAYASHREE PARANJAPE, AND VERONICA RODRIGUES

*Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay 400005, India; and *National Centre for Biological Sciences, TIFR Centre, P. O. Box 1234, Indian Institute of Science Campus, Bangalore 560012, India*

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Drosophila melanogaster larvae and adults respond to a wide range of chemosensory stimuli. We describe the genetics and developmental expression of the *east* gene, mutations which result in adult-specific chemosensory defects. The original isolate of *east* is semidominant for the behavioral phenotype. Several mutations have been generated, some of which are recessive lethals and others that are viable alleles that show a recessive, adult-specific, chemosensory defect. No larval chemosensory defects were observed. The *east* gene is expressed in the neurogenic region at the time of neuroblast segregation and in cells in the peripheral and central nervous system. Our results suggest that *east*$^+$ expression in the nervous system is required for a normal adult chemosensory response and both increases and decreases in levels of the gene product result in a mutant phenotype. © 1992 Academic Press, Inc.

INTRODUCTION

The chemosensory behavior of the fruit fly, *Drosophila melanogaster*, has been well characterized (Barrows, 1907; Begg and Hogben, 1943, 1946; Kikuchi, 1973, Rodrigues and Siddiqi, 1978). Both larvae and adults respond to a variety of stimuli in specific ways (Siddiqi, 1987; Monte *et al.*, 1989; Rodrigues *et al.*, 1991). Adults are attracted to, or repelled by, olfactory and gustatory cues and these responses can be studied in behavior tests that use populations of flies or in "single-fly" assays (Fuyama, 1976; Rodrigues and Siddiqi, 1978; McKenna *et al.*, 1989). Larvae, unlike adults, are attracted to most chemosensory cues, including many that repel adults (Siddiqi, 1987). The chemosensory response is a consequence of the stimulation of the "smell" or "taste" receptors, transduction of this signal, computation of the meaning of the signal in peripheral and more central locations in the insect brain, and, finally, translation into a motor response. While chemoreceptors have not been isolated in *Drosophila*, a recent report (Buck and Axel, 1990) describes mammalian candidate molecules. These molecules resemble G-protein-coupled receptors. Their diversity (Buck and Axel, 1990) suggests that a significant part of the specificity in odor detection could be encoded at the level of primary receptors. Second messenger systems in chemosensory transduction are being studied by *in vitro* methods (Breer *et al.*, 1990). Such approaches, the spatial localization of gene products, and behavioral genetics of mutants should reveal which second messenger routes are used in chemosensation in *Drosophila*. The sensory neurons in the olfactory and gustatory pathways of *Drosophila* make first-order connections in specific regions of the brain; the antennal lobe and the suboesophageal ganglion, respectively (Stocker *et al.*, 1983; Nayak and Singh, 1985). In the antennal lobe discrete structures, called glomeruli, are visible in the light microscope (Venkatesh and Singh, 1984, Pinto *et al.*, 1988; Stocker *et al.*, 1990). "Functional-anatomical" studies, using [2-^{3}H]deoxyglucose to label cells that are active upon olfactory stimulation, show that the olfactory glomeruli are regions of processing of information (Rodrigues, 1988). We know little about the molecular events in this region other than that the neurons in this area of the brain, responding to olfactory stimulation, are likely to be cholinergic (Buchner and Rodrigues, 1984; Buchner *et al.*, 1986).

The diversity of the fly's chemosensory response requires the precise development of the nervous system. The adult sensory neurons are derived from cells located in the imaginal discs of the larva. The choice of cells from this epidermal field that will undergo neurogenesis is under the control of "proneural" and "neurogenic" genes (Simpson, 1990; Campos-Ortega, 1990). Sensory neurons, during pupation, must traverse specific paths to reach their appropriate first-order synapses (Tix *et al.*, 1989; Lienhard and Stocker, 1991). Most of the neurons that will constitute the adult central nervous system are made during the larval stage; there is limited

[1] To whom correspondence should be addressed.

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production of adult neurons during pupation (White and Kankel, 1978; Truman and Bate, 1988). Most studies that describe the changes during metamorphosis that lead to the development of the adult nervous system have been done in the moth *Manduca sexta* and these reports have described the role of hormones and the diverse processes that operate: the reorganization of larval neurons (Levine and Truman, 1985, Truman and Reiss, 1988), the development of adult-specific neurons (Booker and Truman, 1987), and cell death (Weeks and Truman, 1985). Similar processes have been shown to operate in *Drosophila* (Truman, 1990). Mutations that specifically affect aspects of the adult sensory response, but not that of the larva, could identify molecules that are required for the correct development of the adult sensory system or the reorganization of the larval brain during pupation.

We describe the genetics, behavior, and expression pattern of a gene, *east*, and mutations in which lead to adult-specific chemosensory defects. We show that the *east* gene is expressed in the developing larval and adult nervous system. The expression of the gene in the nervous system and the adult specificity of the behavioral phenotype suggest that genetic variations involving this locus could be useful in dissecting the mechanism of adult neural development and function.

MATERIALS AND METHODS

Fly Stocks

The isolation of the "enhancer-trap" strain $east^{P[ry+ETX3]}$, then called ETX3, has been described in Anand *et al.* (1990). Briefly, several strains were established, each carrying a P-element, and integrated at a random chromosomal position. Several of these strains were screened in behavioral assays for chemosensory abnormalities and the chromosomal positions of the enhancer-trap P-element (O'Kane and Gehring, 1987; Bellen *et al.*, 1989a) mapped (Anand *et al.*, 1990). All deficiency and duplication bearing strains were obtained from the Bloomington Stock Center and are described in Lindsley and Zimm (1987). The *daughterless* (*da*)strain was provided by Professor J. A. Campos-Ortega of Cologne, Germany and carried other markers: da^{KX90} *b pr cn bw/CyO*. The markers and balancer chromosome are described in Lindsley and Grell (1968) as are the other markers used in the crosses outlined below.

Chemosensory Response Assays

Odor-induced jump assay. A modification (Ayyub *et al.*, 1990) of the test devised by McKenna *et al.* (1989) was used. Briefly, individual flies were placed in a vertical glass tube and allowed to walk up two thirds the distance to the top. Air was blown through a solution containing the stimulus to deliver a puff of odorant to the flies. A positive response was scored if the flies jumped and hit the bottom of the tube within 5 sec of the initiation of the stimulus. A set of 20 flies was treated as a batch, and the mean and standard deviation of the response was calculated from 10 batches. As a control for the noise level in the tests, the jump response elicited by air blown through water was measured. Wild-type flies were always tested in parallel with the mutants. Tests of statistical significance were performed with the Student's *t* tests.

Adult Y-maze assay. The test was performed as described by Ayyub *et al.*, (1990). Flies were offered a choice between air containing odorant and control air in a Y-maze olfactometer. For benzaldehyde the maze was run vertically for 5 min. The flies which ran into control and odor arms were collected and used to compute the response index (RI). The RI is calculated from the difference of the number of flies in the odor and control arms of the maze divided by the total number of flies taking part in the test. The maze was run four times placed in different orientations for each batch of flies and the average RI calculated. The mean and standard deviation of the response was calculated from 10 independent experiments. Essentially all flies participate in the test.

Feeding preference assays. Adult fly taste behavior was measured as described by Tanimura *et al.* (1982) and Rodrigues *et al.* (1991). All wells of a 60-well microtiter plate were filled with 1% agar; alternate wells also contained 0.2% of the food dye carmoisine red. The stimulus was placed in the uncolored wells for attractant assays and in the colored wells for the assay of response to repellants. Appropriate controls were done to ensure that the agar or the coloring did not influence the interpretation of the results. Two- to 4-day-old flies were starved for 18 hr prior to the test and about 100 flies were introduced into each test plate. The plates were left undisturbed in the dark for about 1 hr, after which they were cooled to immobilize the flies. The color of the flies' abdomens was scored by inspection under a dissection microscope. Control and experimental flies were tested under identical conditions. In each test, 10 observations were taken to calculate the mean and standard deviation. The 10 assays for each experimental point were distributed over 3 days using separate sets of flies. The acceptance of a stimulus is measured by the fraction of flies whose abdomens are uncolored. The tolerance of a stimulus is measured by the fraction of flies with colored abdomens. Reduced activity, if any, of mutant strains would bias the results toward the mutant appearing like the wild type. However, no such reduction in activity by *east* mutants was observed and none

of the strains were apparently sluggish or inactive. Standard controls to eliminate extraneous factors affecting the calculation of the acceptance or tolerance have been detailed in Rodrigues *et al.* (1991) and Balakrishnan and Rodrigues (1991).

Larval chemotaxis. The olfactory responses of larvae to odorants were assayed as described by Monte *et al.* (1989). Third instar larvae were harvested from culture bottles and placed in the center of a petri plate containing 1% agar. Appropriate dilutions of the odorant were placed on a small filter paper disc at one end of the petri plate. The larvae were allowed to distribute themselves for 5 min and counted on the both halves of the plate. The number of animals on the odorant and control halves of the plate were used to calculate the response index which is the difference between the number of larvae in the stimulus and control halves of the plate, divided by the total number of larvae.

Contact chemotactic responses were assayed by dissolving the appropriate dilution of salt in 1% agar. This stimulus containing agar was used to fill half a petri plate. Larvae were placed in the center of the plate and allowed to distribute themselves between the stimulus and nonstimulus containing halves of the plate for half an hour in the dark. The larvae were counted, and the response index was calculated from the difference between the number of larvae in the stimulus and control halves of the plate, divided by the total number of larvae.

A response index of −1 indicates total repulsion and +1 indicates total attraction. Values close to zero reflect a lack of response.

Histology, Immunocytochemistry, and in Situ Hybridization

The expression of the reporter β-galactosidase gene was assayed using the chromogenic reagent X-Gal as described (Wilson *et al.*, 1989, Anand *et al.*, 1990). After staining, dissected larval and adult tissues were washed in 70% ethanol and mounted in 90% glycerol. Eight-micrometer cryostat sections of adult tissue were stained at 37°C overnight, washed extensively with water, dehydrated through a graded alcohol series, and mounted in DPX.

Antibody staining of $east^{P[ry+ETX3]}$ wholemount embryos was carried out by the protocol of A. Gould and R. A. H. White (personal communication) and is detailed in Ashburner (1989). The monoclonal antibody to β-galactosidase was a generous gift from R. A. H. White. The antigen-antibody reaction was revealed using a biotin-avidin-coupled horse radish peroxidase kit (Vector Laboratories). The enzyme reaction was detected with diaminobenzidine (Sigma); embryos were dehydrated through a graded alcohol series and mounted in DPX.

Wholemount RNA *in situs* were carried out in wild-type embryos using a digoxygenin-labeled cDNA clone (pETX3A), by the method of Tautz and Pfeifle (1989).

Isolation of Genomic DNA and cDNA Clones and Molecular Methods

"Plasmid-rescue" of genomic DNA flanking the P-insertion in $east^{ETX3}$ followed the method of Pirrota (1986). Genomic DNA was extracted from 20 to 50 flies, digested with *Hin*dIII or *Sal*I, ligated under conditions that favor monomolecular ligations, and transformed into bacteria that were selected on ampicillin. DNA from colonies that grew under these conditions were analyzed by digestion with appropriate enzymes to ensure that the clones were legitimate. *In situ* hybridization to salivary gland polytene chromosomes verified that the plasmid-rescued DNA was from the site of the P-insertion. *Drosophila* DNA thus isolated was labeled with ^{32}P (Sambrook *et al.*, 1989) and used to screen a wild-type genomic library constructed in the λ-Gem11 vector (Promega, Madison, WI). Positive plaques were purified and phage DNA isolated. DNA prepared from a plaque-purified phage was labeled with biotin-16-dUTP and used as a probe on salivary gland polytene chromosomes for *in situ* hybridization and its hybridization to the 2C1 region confirmed. One positive plaque thus verified, λ-ETX3A, was mapped and the *Sac*I fragments released were labeled with [^{32}P]dATP and used as probe on a *Drosophila* head cDNA library constructed in the λEXLX vector (Palazzolo *et al.*, 1990) by Dr. Bruce Hamilton and kindly provided by him. The cDNA clones are inserted directionally in this vector. Two classes of cDNA clones were isolated from this library. Both clones were similar and hybridized to each other. Both clones hybridized to the 2C1 region of the salivary gland polytene chromosomes where the P-insert also hybridizes. The cDNA clones have been called pETX3A and pETX3B.

One of the cDNA clones, pETX3A, was labeled with ^{32}P and used to screen a RNA blot containing 3 μg electrophoresed poly(A)$^+$ mRNA from different developmental stages.

Generation of Alleles of east by Imprecise Excision

Females homozygous for the $east^{P[ry+ETX3]}$ P-insertion were crossed to males that carried the stable "Δ2-3" transposase source (Robertson *et al.*, 1988). Male progeny of this cross that carried the stable transposase source are identified by the dominant marker, *Stubble* (*Sb*) on the transposase bearing chromosome. These males were crossed to females of the genotype *C(1)RM,y;ry* to identify viable males in which the P-in-

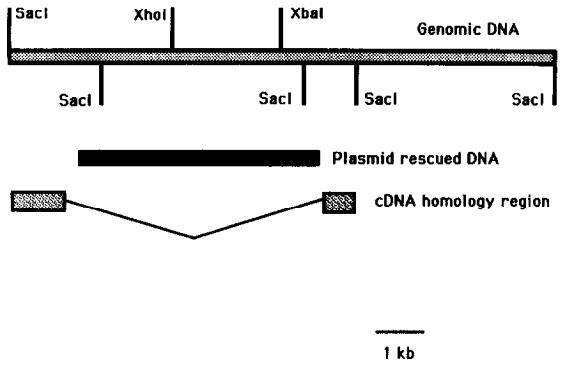


FIG. 1. Restriction map of the genomic clone λ-ETX3A (top), region of homology to the region flanking the P-insertion in *east*$^{P[ry+ETX3]}$ (middle, in black), and region of homology to the cDNA clones (bottom, stippled). The extent of these black and stippled boxes does *not* correspond to the length of these DNAs but indicates the restriction fragments on the genomic DNA, λ-ETX3A, that they hybridize with on Southern blots: The plasmid rescued DNA hybridizes to three of the four *Sac*I fragments and the cDNA clones hybridize to two of the four *Sac*I fragments on λ-ETX3A. The plasmid rescued DNA and the cDNA clones do not hybridize to each other. The scale bar is meant to be used with the restriction map (top) of λ-ETX3A. The sequence of the cDNAs and their restriction map derived from the sequence indicate that the 3′ end of the cDNAs are toward the left of the figure.

sertion has lost the ry^+ marker or to *FM7;ry* females to identify females in which the ry^+ marker had been lost. The cross is designed such that the excision of the P-insertion will take place in the male and therefore no wild-type homologous chromosome will be present. In such a situation excisions that are precise will be very rare events (Engels *et al.*, 1990). However, when P-insertions do not disrupt the protein coding region phenotypic revertants can arise from events that are not precise. As shown in Fig. 1, the *east*$^{P[ry+ETX3]}$ P-element has inserted in an intron. Southern blot analysis of *east*$^{P[ry+ETX3]}$ of some of the strains that reverted the ry^+ eye color marker were done using the entire insert from the lambda genomic DNA clone, λ-ETX3, as a probe. Revertants of the behavioral phenotype show a wild-type restriction pattern (data not shown) for the regions homologous to the cDNA clones in Fig. 1, but show hybridization patterns different from the wild type for the presumed intronic region shown in Fig. 1.

RESULTS

An "Enhancer-Trap" P-Insertion at Chromosomal Position 2C1 Causes a Wide Variety of Chemosensory Defects

A screen for P-element insertion mutants on the X-chromosome identified strains that showed defective chemosensory responses (Anand *et al.*, 1990). We are systematically studying the genetics and molecular properties of these loci. Our initial study of mutant phenotypes examined olfactory responses, rapidly assayed in the "jump test" in which flies jump when exposed to a pulse of odorant (McKenna *et al.*, 1989). Flies that are homozygous or hemizygous for the X-chromosome bearing the P-insertion P[ry$^+$ETX3] (Anand *et al.*, 1990), when examined in this test, show a clear mutant response to olfactory stimulation by several odors (Fig. 2a). The P[ry$^+$ETX3] olfactory defects were initially identified in odor-induced "jump" assays using benzaldehyde and isoamyl acetate as stimulants. P[ry$^+$ETX3] flies are also mutant when tested with ethyl acetate, propionic acid, and butanol (Fig. 2a). While the odor-induced jump assay is convenient and rapid, it can be argued that the olfactory phenotypes seen are a peculiarity of the test. That this is not the case was demonstrated by testing P[ry$^+$ETX3] flies in a Y-maze assay (Rodrigues and Siddiqi, 1978; Ayyub *et al.*, 1990) wherein flies choose between two arms of a maze, the odorant flowing through one arm. Figure 2b shows that P[ry$^+$ETX3] adults have reduced responses to benzaldehyde in this test ($P < 0.02$). We have called the gene defined by the mutant behavioral phenotype *east* and the P-insertion allele is henceforth denoted *east*$^{P[ry+ETX3]}$

The chemosensory defects in *east*$^{P[ry+ETX3]}$ flies are not restricted to olfaction. Males and females of the strain show specific gustatory defects. Wild-type adults are attracted to sugars and also to salt at low concentrations (Tanimura *et al.*, 1982; Arora *et al.*, 1987). These responses are normal in *east*$^{P[ry+ETX3]}$ (Fig. 2c). High salt concentrations normally repel flies (Arora *et al.*, 1987). *east*$^{P[ry+ETX3]}$ mutant flies, however, are more tolerant to high salt concentrations than the wild type (Fig. 2d).

The sensory defects observed in *east*$^{P[ry+ETX3]}$ do not encompass all modalities. The phototactic responses of the adult as measured in the countercurrent test (Benzer, 1967) were normal (data not shown).

The Larval Chemosensory Responses of east$^{P[ry+ETX3]}$ *Are Normal*

Larval olfactory responses can be assayed in a petri plate assay (Monte *et al.*, 1989; Ayyub *et al.*, 1990). The third instar larvae of *east*$^{P(ry+ETX3)}$ show normal attraction responses to the odorant stimuli, isoamyl acetate, propionic acid, and butanol (Fig. 2e). The larvae also show normal contact avoidance of sodium chloride and potassium chloride (Fig. 2f).

east$^{P[ry+ETX3]}$ *Shows a Dominant Chemosensory Phenotype*

When *east*$^{P[ry+ETX3]}$/+ females were tested for their olfactory response in the jump assay they showed a response which was significantly different from that of

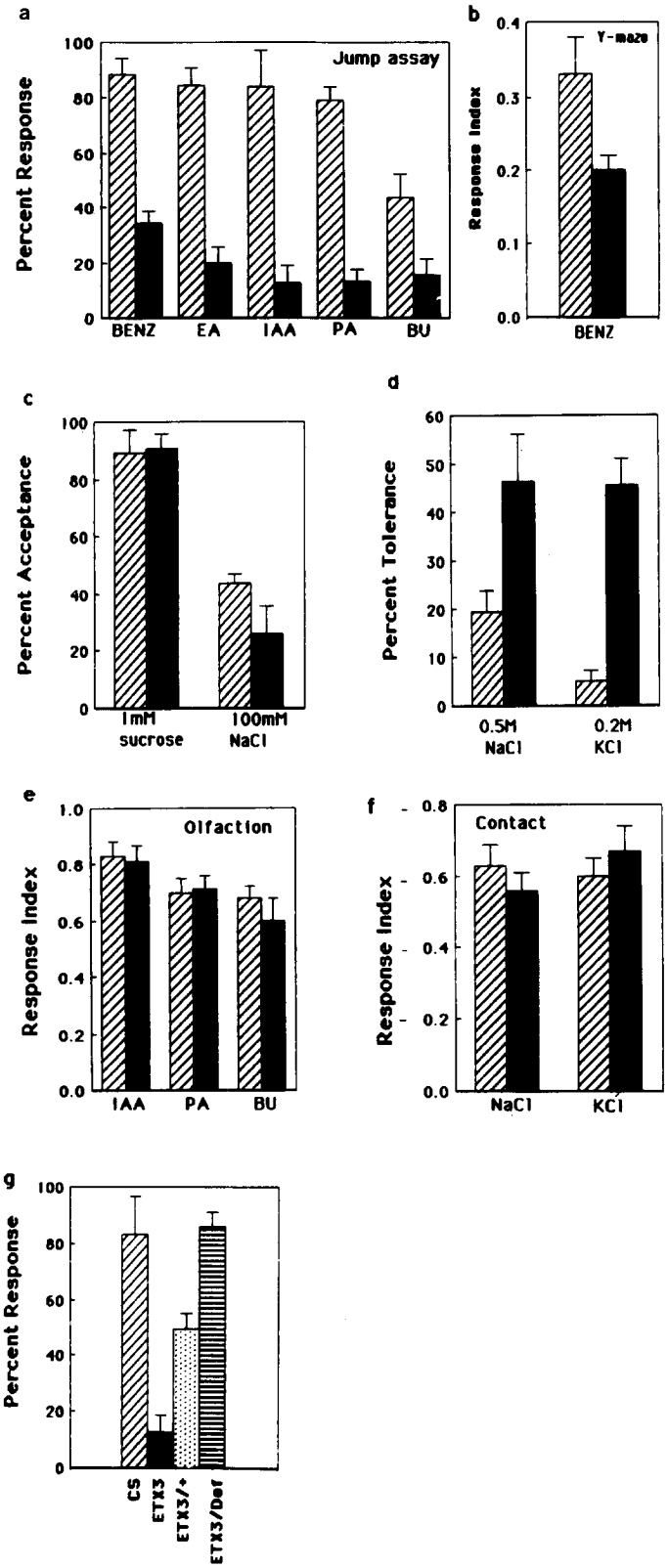


FIG. 2. Behavioral responses of $east^{P[ry+ETX3]}$. In all cases, the columns represent the mean and standard deviations of at least 10 independent experiments. In a–f the first (hatched) column indicates the responses of the wild-type strain (Canton-S) and the second column (filled black) the response of $east^{P[ry+ETX3]}$. (a) Olfactory responses of adults measured in the odor-induced jump assay. (b) Y-maze choice assay. (c and d) Taste responses measured in feeding preference assays to attractants (c) and repellents (d). (e) Olfactory responses of third instar larvae to isoamyl acetate (IAA), propionic acid (PA), and butanol (BU). (f) Contact avoidance of larvae to 0.5 *M* NaCl and 0.5 *M* KCl. (g) Dosage effects of the $east^{P[ry+ETX3]}$ mutation measured in isoamyl acetate-induced jump assays. $P < 0.001$ for $east^{P[ry+ETX3]}/Df(1)dor\text{-}2T$ and $east^{P[ry+ETX3]}/+$; $P = 0.36$ for $east^{P[ry+ETX3]}/Df(1)dor\text{-}2T$ and CS.

the homozygous mutant and the wild-type strain ($P < 0.001$): $east^{P[ry+ETX3]}/east^{P[ry+ETX3]}$ flies show a strong mutant response, $east^{P[ry+ETX3]}/+$ flies, a weaker response compared to wild-type (+/+) flies (Fig. 2g). One possible way to explain this semidominant behavior is that the $east^{P[ry+ETX3]}$ allele is a hypermorph. The P-insert in the $east^{P[ry+ETX3]}$ strain mapped to position 2C1 by *in situ* hybridization of a labeled *lacZ* DNA probe to the salivary gland polytene chromosomes (Anand *et al.*, 1990). If the olfactory defects observed were due to the P-insertion then the behavioral phenotype should also map to the same position. The hypermorphic hypothesis stated above suggests that flies that are $east^{P[ry+ETX3]}$ in *trans* with a deficiency for the 2C1 chromosomal region could show a near wild-type phenotype. This indeed is the case (Fig. 2g). Since the response of $east^{P[ry+ETX3]}/Df(1)dor\text{-}2T$ females are comparable in their jump response to the wild type ($P > 0.3$), this maps the chemosensory defect in the chromosomal region 2B6–2E1, the region uncovered by the deficiency. The response of $east^{P(ry+ETX3)}$ in *trans* with a flanking deficiency, $Df(1)w^{vco}$ (2C5;3C5) is not significantly different from $east^{P(ry+ETX3)}/+$ ($P > 0.4$). The inclusion of the olfactory defect in $Df(1)dor\text{-}2T$ and its exclusion from $Df(1)w^{vco}$ is consistent with the localization by *in situ* hybridization to chromosomal position 2C1. None of the other P-insertion strains we have generated in our experiments and examined for chemosensory defects (Anand *et al.*, 1990) map to this chromosomal position in behavioral tests. In all the behavioral mutants isolated in our laboratory that are presumed to have arisen due to a P-element insertion, the chromosomal location of the insertion and the mapping of the behavioral phenotype are coincident (within the limits of resolution of deficiency mapping). This indicates that it is unlikely that the behavioral phenotype is a consequence of variations in background between strains.

Excisions of the P-Insert from $east^{P[ry+ETX3]}$ Generate Recessive Alleles

The P-element can be excised genetically by crossing $east^{P[ry+ETX3]}$ flies with those that contain a transposase source (Robertson *et al.*, 1989). These crosses were designed so that both viable and homozygous lethal prog-

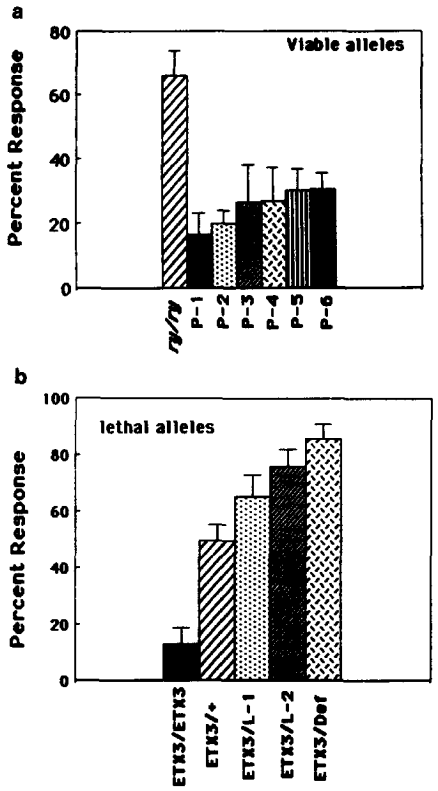


FIG. 3. Odor-induced jump responses of excision-induced alleles of *east* to isoamyl acetate. The columns represent the mean response and standard deviations of 10 independent experiments. (a) The responses of the six viable lines (P1 to P6) are compared to *ry* controls. (b) Lethal excision lines (L1 and L2) assayed in *trans* with $east^{P[ry+ETX3]}$, $P < 0.001$ when $east^{P[ry+ETX3]}$/+ was compared to either $east^{P[ry+ETX3]}$/L-1 or $east^{P(ry+ETX3)}$/L-2. The data for the other combinations shown in this graph are the same data as that shown in Fig. 2g and are shown here again for ease of comparison. The data for Def/+ is 87 ± 4.2 (not shown in graph) and is very close to the value 86.5 ± 4.7 for ETX3/Def (shown in the graph above).

eny of the excisions could be recovered. In most of the homozygous viable strains, the behavioral defects seen in $east^{P[ry+ETX3]}$ were reverted when examined in flies heterozygous or homozygous for the excision chromosome: Of 28 excision lines examined 20 reverted to wild type in behavioral assays for olfaction. The extent of the phenotypic reversion varies between the revertant lines. These results and those from Southern blot analysis of some of the strains that have lost $rosy^+$ marker (see Materials and Methods) demonstrate that the $east^{P[ry+ETX3]}$ phenotype is due to the P-insertion, and the removal of the insert can revert the phenotype.

Six independent viable excision strains were isolated that showed behavioral defects when assayed in odor-induced jump assays against isoamyl acetate (Fig. 3a). The mutant behavior in these strains, designated P1 through P6, was fully recessive. In order to group the alleles into complementation classes, we crossed P2 and P4 to all the other mutant lines. Analysis of the olfactory behavior of the *trans* heterozygotes allowed us to place them into a single complementation group.

The excision crosses yielded five lines that were dead as homozygous females or hemizygous males. Since $east^{P[ry+ETX3]}/Df(1)dor\text{-}2T$ showed normal olfactory responses, we presumed that null alleles at the *east* locus would rescue the hypermorphic behavioral phenotype. Two of the lethal strains, L1 and L2, when tested in *trans* with $east^{P[ry+ETX3]}$ gave olfactory responses which were significantly higher than that of $east^{P[ry+ETX3]}$/+ ($P < 0.001$ for both L1 and L2) (Fig. 3b). These responses can be seen to approach that of $east^{P[ry+ETX3]}/Df(1)dor\text{-}2T$ (Fig. 3b).

In order to test the relationship between the lethals L1 and L2, and the viable complementation class, we tested the olfactory responses of L1/P-2 and L2/P-2. Both these heterozygotes showed reduced responses in the isoamyl acetate jump assay. The phenotype was not significantly different from that of the homozygous P2 strain ($P > 0.1$ for both $east^{L1}$ and $east^{L2}$).The fact that L1 and L2 fail to complement the fully recessive lesion in P2, and the mutants P1–P6 fail to complement each other, suggests that the viable excision mutants are hypomorphic alleles at the *east* locus and L1 and L2 are strains that carry hypomorphic lesions at the *east* locus. We therefore designate the strains P1–P6 as $east^{P1}$–$east^{P6}$. The strains L1 and L2, henceforth designated as $east^{L1}$ and $east^{L2}$, may contain lethal lesions unlinked to *east* or the lethality may be due to complete or near-complete loss of function of the *east* gene. We are unable to choose between these alternatives at present.

The larval olfactory responses of several viable excision alleles were normal (responses of wild-type [CS] = 0.84 ± 0.05; $east^{P3}$ = 0.87 ± 0.05; $east^{P4}$ = 0.84 ± 0.09; $east^{P5}$ = 0.77 ± 0.06; $east^{P6}$ = 0.87 ± 0.05). In addition, we tested the olfactory responses of the larvae of 30 excision lines without bias as to whether these lines had an adult phenotype or not. None of these lines showed defects in larval olfactory behavior ($P > 0.5$), the values for the RI being very similar to the values stated above for the excision lines. This suggests that lesions in the *east* gene result in adult-specific defects and the only larval phenotypes are the early larval lethality seen in $east^{L1}$ and $east^{L2}$. However, at this stage, we cannot exclude the possibility that the larval lethality arises from an independent X-chromosome lesion since we were unable to perform complementation tests between the lethals inter se and between the lethals and a chromosomal deficiency for the region. The one stock bearing a duplication for the 2C1 chromosomal region which would allow such a complementation analysis showed aberrant X-chromosome segregation in control experiments and therefore could not be used.

Molecular Organization of the east Locus

Since the genetics of the 2C1 chromosomal region demonstrated that the *east* gene was required for a normal adult chemosensory response, we initiated a molecular analysis of the gene. The P-element in *east*$^{P[ry+ETX3]}$ carries a plasmid origin of replication and a marker that confers drug resistance to bacteria (Wilson *et al.*, 1989; Bellin *et al.*, 1989a,b). This allows the rapid isolation of *Drosophila* genomic DNA flanking the P-insert by plasmid rescue (Pirrota, 1986). Genomic DNA, thus isolated, was used as a probe to screen a wild-type genomic DNA library and phage clones for the 2C1 region were isolated and verified by chromosome *in situ* hybridization to the salivary gland polytene chromosomes. The *Drosophila* chromosomal DNA insert in the phage was labeled and used as a probe to screen a cDNA library made from mRNA isolated from *Drosophila* heads. Two cDNA clones, pETX3A and pETX3B, were isolated, and their locations verified by chromosome *in situ* hybridization and by Southern blot hybridization to the genomic clone. A restriction map of the genomic clone was generated and the cDNA clones and the plasmid rescued genomic DNA mapped onto the genomic clone. This map is shown in Fig. 1.

Sequence analysis of the cDNA clones revealed that they share common 3′ stretches and differ at their 5′ end; their conceptual translation shows stop codons in all three reading frames and suggests that these clones represent untranslated regions of the mature transcript (C. Mayeda and M. J. Palazzolo, unpublished observations). Northern blot hybridization using labeled cDNA pETX3A as a probe revealed the presence of at least two mRNA species of 9.5 kb and larger (Fig. 4). Both the cDNA clones are much smaller in size (~1.0 kb). The expression of the mRNAs homologous to pETX3A is detected early in embryogenesis, at about 0–6.0 hr and continues during embryogenesis. Levels of the mRNA fall in the last third of embryonic development (16–24 hr) after organogenesis has begun and very low levels of mRNA are seen in the first larval instar. *east* mRNA is not detected on our poly(A)$^+$ mRNA blot in the second larval instar but a faint signal is seen in the third larval instar and during pupal development, which is reproduced poorly in the print (Fig. 4) but is visible in the autoradiographic film. The adult body shows high levels of a 9.5-kb mRNA species and the head has, predominantly, a larger sized mRNA (Fig. 4). Both such transcripts were detected in the early embryo. In addition, Northern blots of mRNA from unfertilized eggs (data not shown) demonstrate the presence of mRNA homologous to pETX3A, corresponding to the 9.5-kb mRNA, indicating a maternal contribution from the gene.

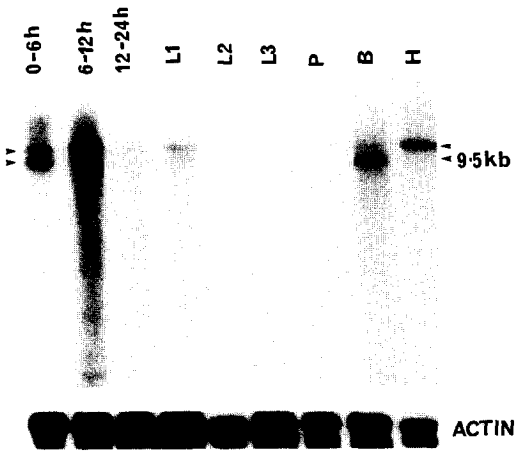


FIG. 4. Northern blot of different developmental stages of wild-type *Drosophila* probed with cDNA pETX3A. Three micrograms of poly(A)$^+$ RNA from different developmental stages of *Drosophila* was loaded per lane. Blots were reprobed with actin cDNA for normalization. The lane representing 6- to 12-hr embryonic RNA shows degradation. Independent blots of these stages showed the presence of two hybridizing mRNA species of >9.0 kb in size resembling the 0- to 6-hr embryonic lane.

Cellular and Developmental Expression of east

The expression of the *east* gene during development was followed in two different ways. The *east*$^{P[ry+ETX3]}$ allele allows the monitoring of gene expression by assaying for β-galactosidase activity encoded by the reporter *LacZ* gene present in the "enhancer-trap" P-insert (O'Kane and Gehring, 1987). Hybridization to mRNA in wholemounts of tissue using digoxygenin-16-dUTP-labeled cDNA, pETX3A, as probes, also allowed the examination of the pattern of expression of the gene. The mRNA expression pattern was similar to that seen by reporter gene assays further strengthening the view that the cDNAs correspond to mRNA from the *east* gene.

east expression is first detected in embryos when the germband is fully extended (stage 9, Campos-Ortega and Hartenstein, 1985). Expression of the *east* gene was detected in regions of the embryo undergoing neurogenesis. Reporter gene activity as well as mRNA expression as detected by hybridization to digoxygenin-labeled cDNA, pETX3A, is seen the laterodorsal region of the procephalic lobe, which will give rise to the supraoesophageal ganglion (Fig. 5A), and the ventral neurogenic ectoderm which contributes to the suboesophageal ganglia as well as the thoracic and abdominal neuromeres (Fig. 5B). *east* mRNA continues to be detected in the presumptive brain and segmental neuromeres during germband retraction in late stage 12 embryos (Fig. 5C). Reporter gene activity (Figs. 5D and 5F) and *east* mRNA (Fig. 5E) continues to be seen in the nervous system

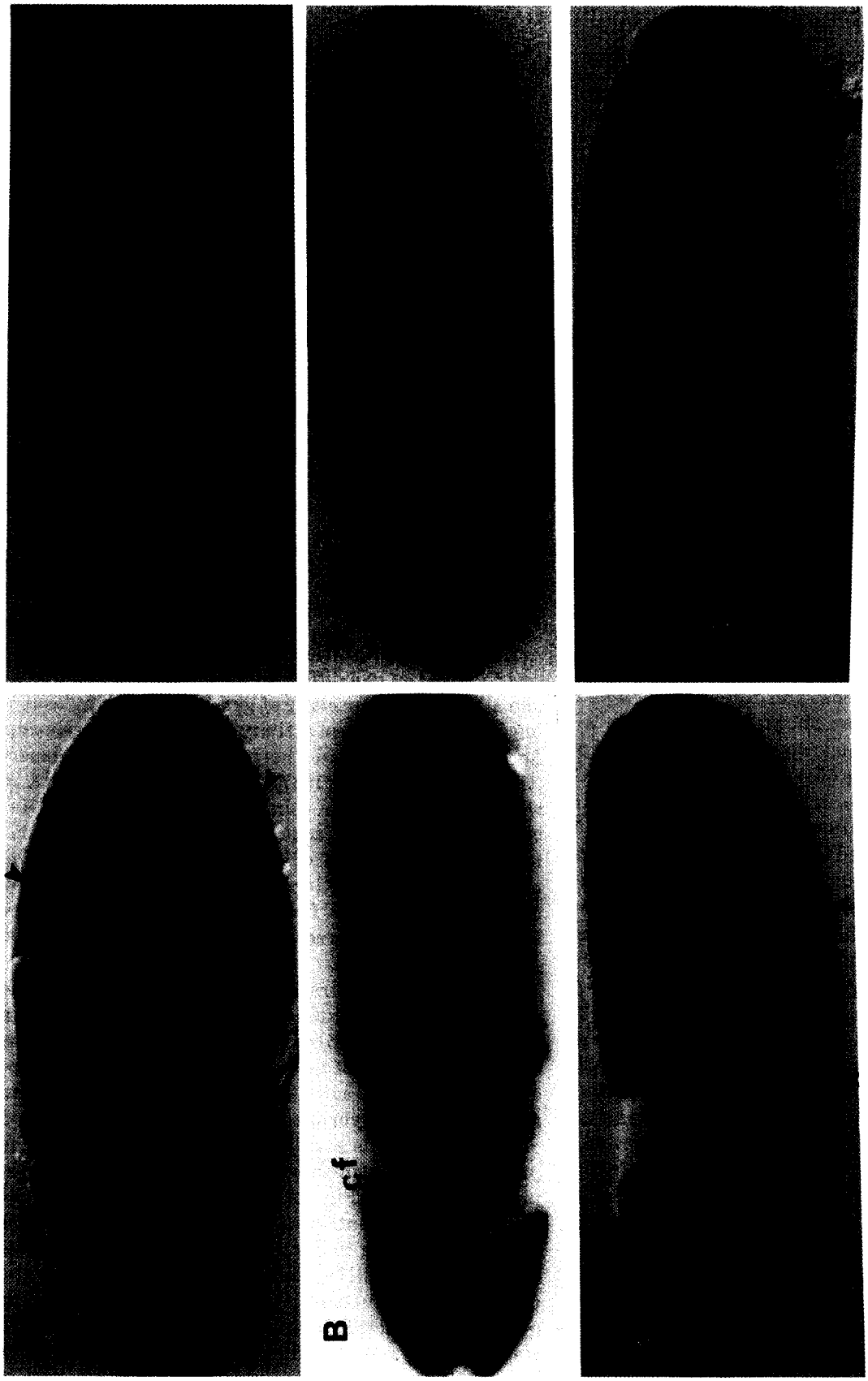
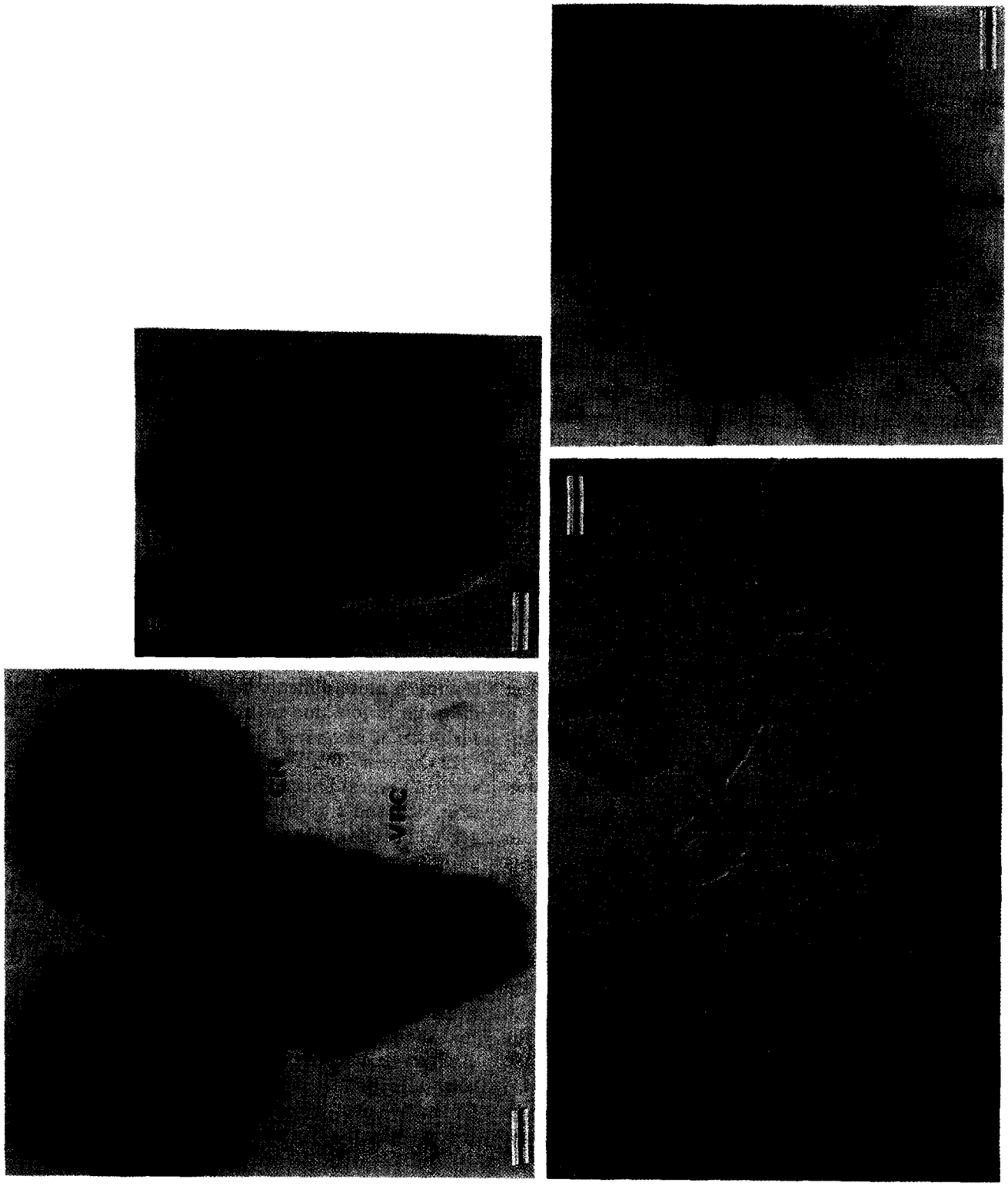


FIG. 5. Expression of *east* in embryos. The pattern of expression of β-galactosidase was followed in *east* embryos using a monoclonal antibody to the *E. coli* enzyme (A, D, F). *east* mRNA was detected in wild-type embryos by *in situ* hybridization with digoxygenin-labeled cDNA pETX3A (B, C, E). (A) Dorsal view of an embryo at the fully extended germband stage (stage 10) showing reporter gene activity in the procephalic neurogenic region (pnr). Staining is also detected in the lateral regions of each segment possibly corresponding to the tracheal placodes (arrowheads). (B) Ventral view of an embryo at stage 10 showing *east* mRNA detected in the segregating neuroblasts of the ventral germband and the procephalic region. cf, cephalic furrow; m, ventral midline. (C) At late stage 12 embryos, mRNA expression can be seen in the neuromeres. (D) After completion of germband shortening at stage 13, reporter activity is detected in the cerebral hemispheres (ch) and in the ventral nerve cord (vnc). (E) Wholemount RNA *in situ* at a similar stage as in (D) showing *east* expression in the supraoesophageal ganglion (spg) and ventral nerve cord. (F) In later (stage 16) embryos strong β-galactosidase activity was observed in the antennomaxillary complex (amx) and the clypeolabrial sense organs (double arrows). Segmentally repeated staining (arrowheads) corresponds to the peripheral sense organs as well as other epidermal cells.


VNC

throughout embryogenesis. The expression is strong in the cerebral hemispheres and shows a segmental pattern in the ventral nerve cord (Figs. 5D and 5E).

Expression of *east* in the other ectodermal derivatives of the embryo is more complex. At the fully extended germband stage (stage 10) a group of nuclei was observed staining laterally in each segment (arrowheads in Fig. 5A). The staining is possibly in the tracheal placodes which also appear at this stage (Hartenstein and Campos-Ortega, 1984). Later, during germband retraction, it is evident that the gene is expressed in the cells of the peripheral nervous system as well as other epidermal cells located near the peripheral sense organs. The staining patterns were compared with those of embryos stained with the neuron-specific monoclonal antibody 22C10 (Zipursky *et al.*, 1984). It was clear that only a subset of the thoracic and abdominal sense organs were stained. In addition to the cells associated with the peripheral sense organs, epidermal cells located on the posterior boundary of each segment were stained (Fig. 5F). In the cephalic region *east* activity is detected in the anterior sense organs. During germband retraction (stage 12), reporter gene activity was associated with the labial, maxillary, and mandibular buds. The staining persists in these areas during head involution and became associated in the fully developed embryo with the antennomaxillary complex and the hypophysis, the epiphysis, and the clypeolabrum (Fig. 5F). As with the trunk sense organs, it is clear that expression occurs in several epidermal cells in addition to the peripheral neurons (Fig. 5F).

Mutations in the *daughterless* (*da*) gene have been shown to remove all peripheral sense organs (Caudy *et al.*, 1988). We examined reporter gene activities in $east^{P[ry+ETX3]};da^{KX90}/da^{KX90}$ embryos. No β-galactosidase activity was seen in the region corresponding to the thoracic and abdominal sense organs. In the anterior regions, labeling in several cells was abolished. However, a few β-galactosidase expressing cells were still observed, suggesting that these were not associated with the sense organs. These results suggest that the segmentally repeated staining in the trunk and some of the strong staining in the anterior of the embryo is, in fact, associated with the sense organs.

The expression of the *east* gene in the larva was monitored by examining reporter gene activity in the $east^{P[ry+ETX3]}$ strain. At the third larval instar, β-galactosidase staining was strong in the cerebral hemispheres as well as in the ventral nerve cord (Fig. 6A). Several of the stained nuclei are probably neuronal. The stained cells in the ventral nerve cord occupy characteristic positions, giving the brain a segmented appearance. A similar pattern of reporter gene activity is seen in pupal brains examined 4 and 8 hr after puparium formation (data not shown).

In the eye-antennal imaginal discs, β-galactosidase activity was observed in the developing ommatidia, posterior to the morphogenetic furrow (Fig. 6B, arrowhead). The staining appeared to be of stronger intensity at the furrow. This was particularly obvious in wholemounts of the eye-antennal disc probed with digoxygenin-labeled *east* cDNA pETX3A (data not shown). The staining was shown to be associated with the morphogenetic furrow, since progressively older discs showed movement of the zone of mRNA expression with the migrating morphogenetic furrow.

The observed reporter gene activity in the larval stages contrasts with our detection of only low level of *east* mRNA on Northern blots during larval development (Fig. 4). This could be the consequence of aspects of regulation of *east* that the reporter gene in the enhancer-trap allele does not reflect. Alternatively, the large size of the *east* mRNA may make detection in Northern blots difficult if the steady-state level of the message is low due to instability and/or lower transcription as compared to other stages.

In the $east^{P[ry+ETX3]}$ adult β-galactosidase activity was seen in several but not all neuronal nuclei in the brain (Fig. 6C) and the thoracic ganglion. Staining was observed in cells of the retina and the proboscis, and weak staining is detectable in the antenna. In each of the sensilla of the proboscis, at least three cells express β-galactosidase (Fig. 6D, arrow). X-Gal-stained wholemounts of the proboscis of $east^{P[ry+ETX3]}$ were compared to wholemount probosces stained with the 22C10 monoclonal antibody. The position of the stained nuclei suggests that they belong to taste neurons. Previous studies had shown that a typical taste sensillum on the proboscis is composed of five neurons and four associated accessory cells (Falk *et al.*, 1976; Nayak and Singh 1983, 1985). Of the five neurons innervating a single sensillum, four are chemosensory and one is mechanosensory (Falk *et*

FIG. 6. *east* expression in third instar larvae and adults. (A) Third instar $east^{P[ry+ETX3]}$ larval brain stained with the chromogenic substrate X-Gal. Strong enzyme activity was detected in the cerebral hemispheres (ch) and ventral nerve cord (vnc). (B) Eye disc at the third larval instar stage. β-galactosidase staining is seen posterior to the morphogenetic furrow (arrowhead). (C) Horizontal, 8 μm, cryostat section through the head of $east^{P[ry+ETX3]}$ adult stained with X-Gal. Stained nuclei are detected in the retina (r), lamina (l), medulla (m), and suboesophageal ganglion. The antenna (ant) are weakly stained. The staining is expected to be nuclear localized but is, in fact, diffused. This happens reproducibly in this tissue. (D) Wholemount of proboscis showing staining in a subset of neuronal cells (arrow) innervating each taste sensillum (ts). Some nuclei which are associated with the taste nerve (arrowheads) are stained. Scale bar in (A) 90 μm, (B) 120 μm, (C) 100 μm, and (D) 50 μm.

al., 1976). The four gustatory neurons have distinct functional specificities, one responds to sugars, one to water, one to salts at low concentrations, and one to salts at high concentrations (Rodrigues and Siddiqi, 1978; Fujishiro *et al.*, 1984). The labellar neurons project to defined regions in the suboesophageal ganglion (Fig. 6C). In addition stained nuclei which possibly belong to glial cells are observed closely associated with the taste nerve (arrowheads in Fig. 6D).

DISCUSSION

A P-element insertion in the 2C1 chromosomal region causes abnormal responses to chemosensory cues in adult *Drosophila*. Behavioral genetics of the P-insertion strain and of mutants generated by imprecise excision of the element define a locus we call *east*. The original isolate, $east^{P[ry+ETX3]}$, is a hypermorph. This is demonstrated by the semidominant nature of the mutation and the wild-type behavior of flies of the genotype $east^{P[ry+ETX3]}/Df(1)dor\text{-}2T$. This property of $east^{P[ry+ETX3]}$ allowed the isolation of putative null alleles, $east^{L1}$ and $east^{L2}$. Certain viable alleles of *east* isolated show a recessive olfactory response. All *east* alleles, both recessive and dominant show adult-specific chemosensory defects. None of the recessive viable alleles nor over 30 lines of excision strains isolated without bias toward their adult phenotype showed any larval behavioral defect. These results would imply that both increase and decrease of the *east* gene product affect adult chemosensory behavior.

The sensory system of the adult fly is synthesized during pupation. Sensory neurons from everting imaginal discs traverse specific tracts and synapse with central targets (Jan *et al.*, 1985; Stocker and Gendre, 1988; Tix *et al.*, 1989). Thus, adult-specific phenotypes could result from defects in sensory development. We do not know if this is the cause of the mutant behavior observed in *east*. The regions of the antennal or labial disc that give rise to imaginal sensory structures do not stain for reporter gene activity in the third larval instar. The development of neuronal lineages in the antennal disc takes place later during development, the earliest neurons being visible 3 hr after puparium formation (Lienhard and Stocker, 1991). In the labial disc, which gives rise to the proboscis, the first neurons are visible only after puparium formation (K. Ray, personal communication). The analysis of *east* expression in the developing pupal antennal and labial discs is thus required to determine if defects in neuronal lineages from these discs cause the mutant phenotype. The recent study of the development of the antennal disc by Lienhard and Stocker (1991) and of the labial disc (K. Ray, V. Hartenstein, and V. Rodrigues, in preparation) now allow comparisons between mutant and wild-type development to be made. Although reporter gene activity is detected in the developing eye disc, we do not see any gross defect in the compound eyes nor any phototaxis defect in *east* mutants in the relatively crude phototaxis tests done.

Adult-specific phenotypes could also result from more central requirements for the gene during development. During embryonic development neuroblasts are segregated that give rise to the larval nervous system (Hartenstein and Campos-Ortega, 1984). The ventral nervous system of the adult is likely to include many neurons present in the larval ventral ganglion (Truman and Bate, 1988). In addition neuroblasts are present during the larval stage, in the CNS, that will also give rise to adult neurons. Truman and Bate's (1988) experiments show that only about 7% of the adult nervous system is derived from larval neurons synthesized during embryogenesis, the rest arising from the division of neuroblasts present in the larva and undergoing mitosis during larval development. However, the embryonically derived neurons are likely to play an important role in the development of the adult nervous system, as they could serve as paths that developing adult neurons use to construct the adult CNS (Thomas *et al.*, 1984). One of the best documented changes in the nervous system of *Drosophila* during metamorphosis is that of the changes in the reorganization of the optic lobes and the mushroom bodies (Truman, 1990; Technau and Heisenberg, 1982). The mushroom bodies are thought to be involved in the processing of peripheral sensory inputs and in processes such as memory and learning. At the peripheral level, in the olfactory system, the formation of the antennal glomeruli in the adult is a consequence of interactions between incoming sensory fibers of ectodermal origin and the glia, of mesodermal origin (Pinto *et al.*, 1988, Lienhard and Stocker, 1991). Both the reorganization of the mushroom bodies and the formation of the antennal glomeruli are examples of postembryonic changes that must be correlated with adult-specific chemosensory behavioral responses.

The pattern of reporter gene expression in $east^{P[ry+ETX3]}$ in the embryo, larva, and adult indicates that *east* is expressed in a large part of the central nervous system during development. Developmental studies on the lethal alleles of *east* may provide information on the role of the gene in the developing CNS. In addition, the isolation of alleles of *east* by chemical mutagenesis will also serve to define the functions of the locus. We have isolated two cDNA clones from the *east* region. When used as a probe to detect mRNA in wholemounts of wild-type embryos, the cDNA pETX3A reveals a signal very similar to that seen when reporter gene expression is examined in $east^{P[ry+ETX3]}$ embryos. This suggests that the cDNA clones correspond to *east*

mRNA. Sequence analysis of both the cDNA clones shows that they represent untranslated regions of the mRNA (C. Mayeda and M. J. Palazzolo, personal communication). The untranslated region of mRNAs are thought to have roles in regulating the stability of these molecules (Malter, 1989). The long, presumably 3′, untranslated sequence in the *east* mRNA could have a destabilizing role and thus regulate levels of the *east* product. If this is the case, one way in which a P-insertion could result in a hypermorphic phenotype is that the P-insert in $east^{P[ry+ETX3]}$ could result in a message that is more stable than the wild-type *east* mRNA and result in the synthesis of higher level of the gene product.

The alleles $east^{L1}$ and $east^{L2}$ are dead as homozygotes or hemizygotes. The pattern of expression of both the reporter gene and mRNA shows that the gene is active during embryogenesis as early as the beginning of germband extension. However, the lethal period is later in development. This implies that redundancy of early function allows survival through embryogenesis, or that $east^{L1}$ and $east^{L2}$ do not represent completely null mutants for the early functions. One of the *east* transcripts is present in unfertilized eggs (the 9.5-kb transcript in Fig. 4). This maternal contribution could allow survival of the lethals through embryogenesis. Studies on the phenotype of *east* lethals in animals where the maternal contribution has been removed will allow the complete loss of function phenotype to be assayed. The mutant *smellblind* (Lily and Carlson, 1990; Rodrigues and Siddiqi, 1978) is another example of a "chemosensory" gene that has a vital role (Lily and Carlson, 1990).

The enhancer-trap approach (O'Kane and Gehring, 1987) and its rapid facility for isolating cDNAs that represent mRNA coding regions in the vicinity of the P-insertion (Wilson *et al.*, 1989; Bellin *et al.*, 1989a,b; Bier *et al.*, 1989; Doe *et al.*, 1991) allow the *in situ* assay of gene activity by expression of the reporter gene and the verification that the mRNA pattern of expression is similar. Our experiments show clearly that the *east* gene is expressed in both the sensory and central nervous system during development. The expression in the PNS in the wild-type embryo is confirmed by absence of expression in these regions in the background of the *da* mutation. This predominant expression of the gene in the nervous system during development leads us to believe that the behavioral phenotypes of *east* mutants most probably result from requirements of the wild-type product in the nervous system.

A major problem in the molecular analysis of the nervous system is determining the function of the many thousands of mRNA molecules that are expressed in this tissue (Palazzolo *et al.*, 1989). Assigning function is made easier if the amino acid sequence of the protein that the mRNA conceptually translates suggests a particular role because of its homology to already characterized motifs. The spatial localization of specific mRNAs may also suggest roles if expression is restricted to cells whose functions are known or can be deduced (Doe *et al.*, 1991). In some instances, patterns of expression of the molecule are not always revealing of function. The early zygotic expression of the *da* gene product is ubiquitous but it has very specific effects on the nervous system (Caudy *et al.*, 1988). While genetic analysis can provide clues to function, it is clear that gene products often have roles in many different pathways and stages, some of these roles being essential for viability. The loss of function phenotype of many genes is likely to be death, thereby making the analysis of later functions impossible. In yet other cases redundancies in the pathways may pose difficulties in the analysis of function.

We have been using the chemosensory pathway as a sensitive assay for the study of molecules that are expressed in the nervous system. The behavioral repertoire of the fly in response to chemical cues is diverse and subtle. This property in conjunction with the sensitivity of the response allows the detection of both quantitative and qualitative alterations in behavior. We expect that behavioral screens that test the animal's response to chemosensory cues can detect subtle effects of mutations that anatomical inspection may not readily reveal. In conjunction with a molecular analysis, chemosensory genetics can often provide clues to function of well studied molecules in the cellular context of their domains of expression, such as the type A potassium channel encoded by the *Shaker* locus (Balakrishnan and Rodrigues, 1991) and also identify new molecules (Hasan, 1989; Campbell *et al.*, 1992), and provide a functional assay by which their expression in the nervous system can be studied. The experiments with *east* are part of our efforts to dissect the function of genes in diverse processes by examining their roles at levels from the molecular to behavioral.

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