Genotypic and Phenotypic Characterization of the *Drosophila melanogaster* Olfactory Mutation *Indifferent*

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ABSTRACT

Two *Drosophila melanogaster* third chromosomes carrying the EMS-induced mutations *IndifferentA* (*IndfA*) and *IndifferentB* (*IndfB*), previously isolated from larvae showing an anosmia when stimulated with nonanol, were recombined with a multi-marked chromosome in order to localize the mutant character(s). Recombinant strains were tested for their larval olfactory responses and classed as either mutant or wild type; both *Indf* characters were found to be located on the right arm of the chromosome, between *ebony* and *claret*. Deletion mapping suggests that the *Indifferent* wild-type character is a haplo-insufficiency and that *IndfA* and *IndfB* are located in cytological region 96A2-7. Deficiencies and both mutant strains were tested with 14 closely related odors (alcohols, acetates, acids and methyl esters, between eight and 10 carbons long). When stimulated with methyl octanoate, *IndfA* and *IndfB* appeared recessive; noncomplementation was observed for this phenotype in *IndfA/IndfB* hybrids indicating that the two characters are allelic. The overall responses of *IndfA*, *IndfB* and the deficiencies indicate that *Indf* is involved in processing organic odors of between eight and 10 carbons in length.

GENETICS has contributed substantially to the study of olfaction. Most spectacularly, molecular genetic techniques have been used to isolate super-families of several hundred genes that apparently code for vertebrate olfactory receptor proteins, based on homology with other G-coupled receptors (BUCK and AXEL 1991). This has led to the localization of different classes of receptor neurons within the olfactory epithelium (NEF *et al.* 1992; NGAI *et al.* 1993) and their correlated olfactory glomeruli (RESSLER *et al.* 1994; VASSAR *et al.* 1994). These studies have also strongly reinforced the hypothesis that each receptor neuron expresses only a single receptor type (CHESS *et al.* 1994).

Studies using the "enhancer-trap" technique in Drosophila have led to the isolation of genes that are expressed in olfactory organs (RIESGO-ESCOVAR *et al.* 1992; RAHA and CARLSON 1994), but the specific role of given receptor molecules or even neurons remains unknown. Single receptors can respond to more than one odorant (SICARD and HOLLEY 1984), so the relationship between receptors and odorants may not be very specific: when introduced into a bacillovirus vector, one of the putative receptor proteins only produced a relatively small and unspecific response following olfactory stimulation (RAMING *et al.* 1993). While this result may merely indicate that the key stimulus for this particular receptor protein has not yet been found, it seems probable that the olfactory system's ability to detect thousands of different stimuli is not simply due to the existence of a very large number of highly specific receptor proteins, but is rather the result of complex integrative functions carried out by glomeruli or similar structures on the basis of "odor primitives" (chemical function, chain length, etc.) detected by separate receptor neurons which, taken together, constitute the "sensory image" of the odor (SHEPHERD 1994).

One way of investigating specific olfactory responses, and thus the relation of odors and receptors, is to study genetic anosmias. Organisms that are unable to detect one or a group of odors may lack a specific element of the odor detection pathway. By determining the phenotype (specific anosmia) and the genotype (in the best of circumstances, the sequence), it may be possible to show the relationship between the two, thus elucidating both specific and general aspects of olfactory function. Such a result has recently been found for *Caenorhabditis elegans*, where the *odr-10* gene codes a receptor required for wild-type olfactory responses to diacetyl (SENGUPTA *et al.* 1996).

A large number of olfactory mutants are known in *Drosophila melanogaster* (see CARLSON 1996 for a review) but none of them have yet fulfilled their promise of revealing a specific relationship between phenotype and genotype. Many of these mutants do not show complete anosmias but aberrant or partial responses, or altered thresholds. Others are highly pleiotropic. Thus *olfD*, a mutation that was isolated on the basis of a reduction in its responses to ethyl acetate, benzaldehyde and butanol and the absence of a response to iso-amyl acetate (RODRIGUES and SIDDIQI 1978; AYYUB *et al.* 1990) turns out to be allelic to *smellblind*, a previously described olfactory mutant (LILLY and CARLSON 1990),

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which also shows interactions with *paralytic,* a gene that encodes a Na^+ channel that is not specific to the olfactory system (LILLY *et al.* 1994). An interesting example of such plieotropy is the morphological mutation *Scutoid,* which also specifically decreases olfactory responses to short-chain acetate esters and ketones (DUBIN *et al.* 1995). DNA sequencing has not necessarily led to enlightenment. *olfE,* a mutation that shows aberrant responses to acetates, does not code for a transmembrane receptor protein and is expressed throughout the early embryo, indicating that the mutation is not specific to olfaction (HASAN 1990). Another mutant, isolated on the basis of a reduced response to benzaldehyde, turns out to be an allele of *pentagon,* a gene affecting pigmentation (HELFAND and CARLSON 1989). Furthermore, the exact nature of the olfactory phenotype of such mutants has generally been subject to relatively limited study with only a few odors.

The *Indifferent* mutants are anosmic when stimulated with the odorant nonanol, while responses to nine other alcohols are reported to be normal (COBB *et al.* 1992). Nonanol induces repulsion in wild-type adults and larvae at all doses tested, with larvae showing a slight dose-response curve (COBB *et al.* 1992). Three mutant strains (IndfA, IndfB and IndfC; the first and last are reported to be semidominant, IndfB is dominant) were isolated following EMS mutagenesis of chromosome *3* using a larval olfactory test. Larvae were chosen because of their simple olfactory system and the relative ease with which olfactory responses can be tested. Each of the larva's two olfactory organs only contains 21 neurons, and there are no known glomeruli or similar integrative structures in the larval brain. By contrast the adult fly has ~1200 neurons in each antenna and the fly brain contains 22 glomeruli (see STOCKER 1994 for a review).

Because of the dominance or semidominance shown by the three Indf strains, it is not yet known whether the mutations are allelic (COBB *et al.* 1992). The three strains show slightly different phenotypes when stimulated with nonanol, IndfA being completely anosmic, while IndfB and IndfC show wild-type responses to high and low levels of nonanol, respectively (COBB *et al.* 1992). These strains also show abnormal responses to heptyl acetate (COBB and DANNET 1994), suggesting that the mutation(s) affect some common element involved in the detection of nonanol and heptyl acetate. This article presents the cytological localization and further genotypic and phenotypic analysis of two of the Indf mutations, *IndfA* and *IndfB.*

MATERIALS AND METHODS

Canton-S (CS): A laboratory strain originally from the USA was used as a wild-type reference strain throughout.

IndifferentA (IndfA) and IndifferentB (IndfB): Olfactory mutations induced by EMS mutagenesis of the CS strain (COBB *et al.* 1992). *IndfA* is semidominant over CS, *IndfB* is dominant (COBB *et al.* 1992). Both strains are homozygous viable and, unless otherwise stated, all *Indf* larvae tested were homozygous.

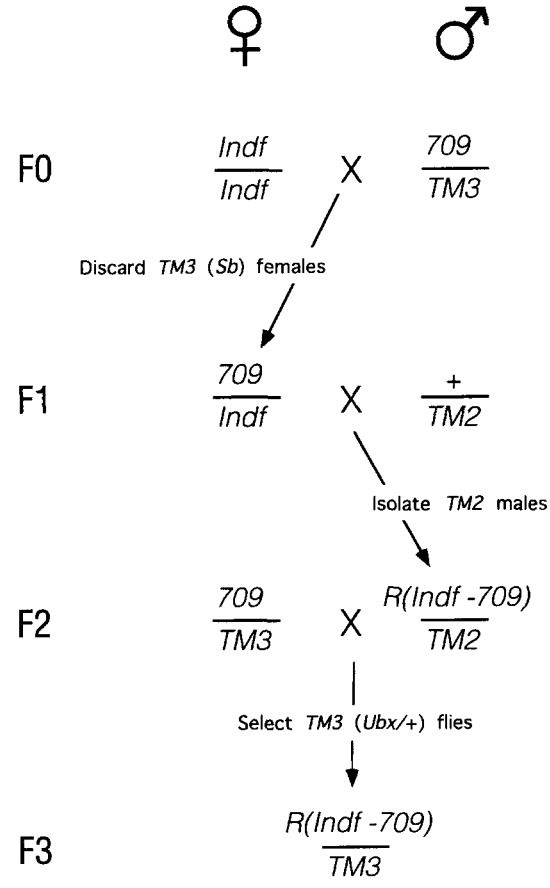


FIGURE 1.—Crossing scheme for the isolation of recombinant *Indf/ 709* chromosomes. F_2 males potentially recombinant for the *709* marker chromosome and *IndfA* or *IndfB* [*R(Indf-709)*] were isolated and each crossed with four *709/ TM3* females. Male and female offspring from these crosses carrying *TM3* and the potentially recombinant chromosome were crossed, producing a stable recombinant line *R(Indf-709)/ TM3.* Each line was then scored for its 709 marker phenotype and those lines showing recombination with the *Indf* chromosome were scored for their nonanol olfactory phenotype. This procedure was done for both *IndfA* and *IndfB* chromosomes.

709: A strain carrying the *709* multimarked chromosome *3* (*ru, h, th, st, cu, sr, e, ca*) balanced by *TM3 (Sb, Ser).* With the exception of *ebony,* these markers are only observable in adult flies.

slowpoke (slo): A mutation affecting calcium-activated potassium channels, genetic location 3:85.0, cytological location: 96A17 (ATKINSON *et al.* 1991).

Deficiencies: These are listed in Table 3. All marker and deletion stocks obtained were from the Umea (Finland) Drosophila stock center, with the exception of *slo, Df(3R)slo8* and *T(Y;3)A117,* which were gifts from Dr. B. GANETZKY.

Recombinant lines: Recombinant chromosomes between the 709 marked chromosome *3* and IndfA and IndfB were created and cloned according to the protocol in Figure 1, creating isomale recombinant lines. This procedure was adopted in preference to noting the olfactory responses of larvae carrying unknown recombinant chromosomes and then recording the marker phenotypes of the eclosed adults,

as had previously been done for sex-linked genes (COBB and DANNET 1994) because preliminary experiments revealed that there was a differential mortality associated with certain combinations of markers and balancer chromosomes, thus producing a bias in the results. This effect may be due to the *Indf* chromosomes, to other factors in the genetic background of these strains, or to factors on the marker and balancer chromosomes, or to all of the above.

Hybrids between IndfA, IndfB and CS: Virgin male and female flies were allowed to mate, and the resultant hybrid larvae were collected. Reciprocal hybrids were made for each cross and were tested; no significant differences were found between reciprocal crosses in any case, so data were systematically pooled. All strains were reared on normal Drosophila medium and kept at 25 ± 0.5° on a 12:12 light:dark cycle.

Olfactory tests: A modified version of ACEVES-PIÑA and QUINN's (1979) olfactory plate test was used, as described in COBB *et al.* (1992). Adult females were allowed to lay eggs overnight on a 2.5% agar medium (1% acetic acid and 2% ethanol). Nine hours after the end of egg-laying a thick yeast paste was provided for food. After 18–22 hr of feeding, larvae were tested in the olfactory paradigm. Larval olfactory responses are strongest at this age (COBB *et al.* 1992; COBB and DANNET 1994). Prior to testing, larvae were washed from the yeast paste and starved on a clean agar dish for 1 hr. Between 15 and 60 larvae were placed in the center of a petri dish covered with 10 ml of 2.5% agar. On either side of the petri dish, a 1-cm circle of filter paper was placed on the agar. Undiluted odorant [1 μl, except for hexyl acetate (0.1 μl) and heptyl acetate (0.5 μl)] was loaded onto one of the filter papers. The lid of the dish was replaced, and after 5 min the numbers of larvae on either side of the dish were noted, together with the number of the larvae which did not "choose" either side. A response index (*RI*) was calculated for each dish [(n odor − n control/n total) × 100]. This index varies between −100 (total repulsion) and +100 (total attraction). Stimulus/control sides were rotated from dish to dish. Means and standard errors were calculated on the basis of the number of dishes observed (≥8) for each point. All chemicals were synthesis grade and were from Merck or Sigma. This test does not involve gustation (COBB *et al.* 1992) and the presence of relatively large numbers of larvae does not produce a "stampede effect" (MONTE *et al.* 1989). Dose-response curves have been shown to exist for wild-type responses to the acetates and alcohols studied here (COBB *et al.* 1992; COBB and DANNET 1994).

RESULTS

Distinguishing the wild-type and Indifferent nonanol phenotypes: The responses of CS wild-type and homozygous *IndfA* and *IndfB* mutant larvae to nonanol were sampled repeatedly (85, 93 and 71 times, respectively). CS larvae were repulsed by nonanol (mean $RI = -36.16 \pm 2.19$), while both *IndfA* and *IndfB* larvae were indifferent ($RI = -0.10 \pm 1.87$ and -3.65 ± 2.08, respectively.) The two *Indifferent* strains did not differ from each other ($t = 1.27$, d.f. = 162, P = NS) and both were highly significantly different from CS ($F_{2,248} = 96.57$, $P = 0.0001$). However, the distribution of wild-type response indices overlaps with those of the two mutant strains (Figure 2). In order to allow any given olfactory plate test to be allocated to one of the two phenotypes, an empirical cut-off point of −20 was established. This value would give rise to a misclassification in around

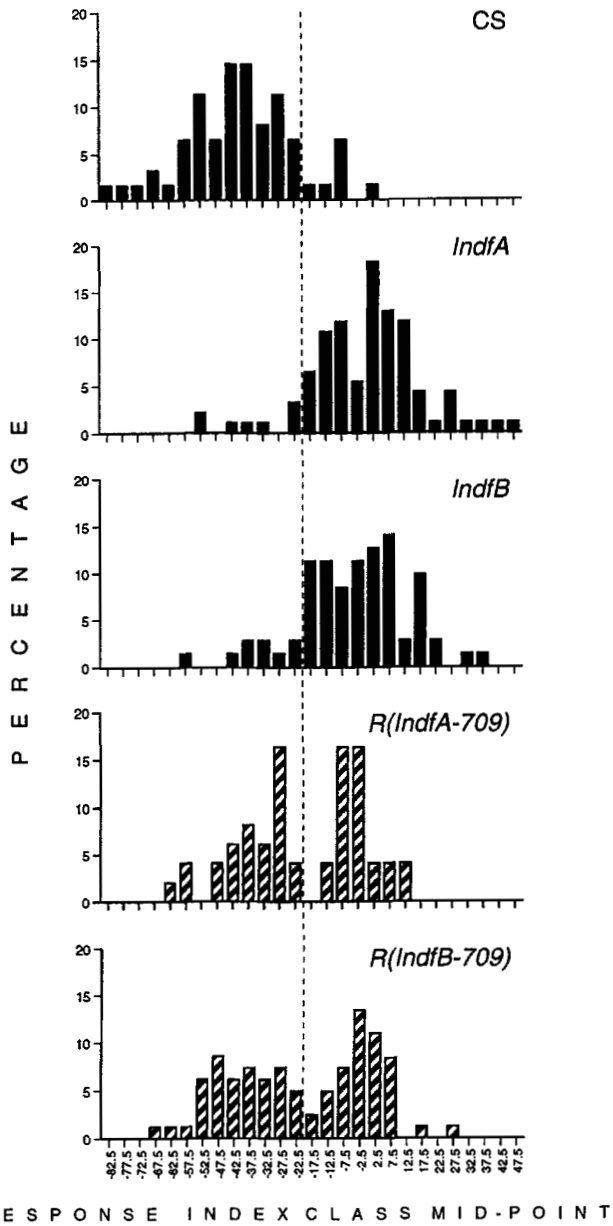


FIGURE 2.—Percentage frequency histograms of olfactory response indices for CS, *IndfA*, *IndfB* and strains recombinant for the *709* marker chromosome and *IndfA* [*R(IndfA-709)*] or *IndfB* [*R(IndfB-709)*] following stimulation with nonanol. Response indices for CS, *IndfA* and *IndfB* (black bars) were from individual olfactory plate tests (n = 83, 93 and 71, respectively). Response indices for the two classes of recombinant strains (hatched bars) were mean scores for each strain, each based on up to 12 olfactory plate tests. Forty-nine *R(IndfA-709)* lines and 82 *R(IndfB-709)* lines were tested. Dotted vertical line indicates the empirical cut-off point (RI = −20) established upon inspection of the data for CS, *IndfA* and *IndfB* to distinguish the wild-type and mutant nonanol olfactory responses. For further details, see text.

10% of observations (11.3% for CS, 8.6% for IndfA and 9.9% for IndfB). It should be noted that these misclassification probabilities are for a sample size of n = 1. The probability of misclassification for a given line can be easily reduced by making several observations.

This kind of procedure has previously been used to distinguish overlapping phenotypes (FERVEUR 1990).

Localizing the Indf mutations by recombination: Isomale recombinant lines were created between the *709* multimarked chromosome and the *IndfA* and *IndfB* chromosomes (*R(Indf-709)*), following the protocol shown in Figure 1. In most cases, vigorous homozygous recombinant lines could not be created because of the deleterious effects of the markers. *R(Indf-709)* chromosomes were therefore maintained against the *TM3* balancer chromosome. In such strains the larvae were a mixture of *R(Indf-709)* homozygotes and *R(Indf-709)/TM3* heterozygotes. In order to test whether *TM3* had an effect on the nonanol olfactory phenotype, *R(Indf-709)/TM3* heterozygotes and their equivalent *R(Indf-709)* homozygotes were compared for their responses to nonanol. Four IndfA and four IndfB *R(Indf-709)* chromosomes were tested, two wild type and two showing a mutant nonanol phenotype in each case, in both the homo- and heterozygous state. For both IndfA and IndfB, there was no significant difference between homo- and heterozygotes ($F_{1,30}$ = 1.23 and 0.57, respectively, P = NS), nor was there any interaction between genotype and nonanol phenotype ($F_{1,30}$ = 0.22 and 3.90, respectively, P = NS), showing that *TM3* produced a wild-type nonanol phenotype and that data from homozygotes and heterozygotes could thus be pooled.

Forty-nine *R(IndfA-709)* and 82 *R(IndfB-709)* recombinant lines were tested. Although these are small sample sizes in terms of recombinant mapping, the olfactory phenotype of each line was reliably determined by testing each line up to 12 times. Sixty-eight percent of lines were tested at least three times. Five lines could only be tested once, due to viability problems. The frequency distributions of the *RI*s for these two sets of recombinants are shown in Figure 2. Both experiments gave a bimodal distribution, with the two groups differentiated by the cut-off point of −20. The narrower distributions for both phenotypes in the recombinant lines as compared to the data for CS, IndfA and IndfB in Figure 2 are due to the fact that the response index for each *R(Indf-709)* line is a mean score of ≤12 separate olfactory plate tests. The two phenotypic groups (mutant and wild type) were of approximately equal size in both experiments and had mean values that were not significantly different from those found for the original distributions (Table 1), indicating that no other characters apart from those on chromosome *3* are involved in the nonanol olfactory phenotype.

In order to arrive at a rough localization of *IndfA* and *IndfB*, a series of ANOVAs were carried out on the response indices for recombinant strains showing different combinations of six pairs of morphological markers (*ru h, h st, st cu, cu sr, sr e, e ca*), from left to right on the chromosome. For each pair of markers, response indices were compared for strains showing all four combinations of wild type and mutant markers (+ +, + −, − +, − −). For both *IndfA* and *IndfB*, only *ebony* (*e*) and *claret* (*ca*) revealed significant differences between the markers and the wild types. *IndfA* showed significant effects of *e* ($F_{1,45}$ = 10.47, P = 0.002), *ca* ($F_{1,45}$ = 14.62, $P < 0.001$) and a significant interaction ($F_{1,45}$ = 6.43, P = 0.015), whereas *IndfB* only showed an effect of *ca* ($F_{1,78}$ = 7.23, $P < 0.01$) and a barely significant interaction ($F_{1,78}$ = 4.24, P = 0.043).

TABLE 1

Responses to nonanol of recombinant strains

Genotype	Nonanol phenotype	*RI*
R(IndfA/709)	+	−36.82 ± 2.29 (25)
	−	−2.66 ± 1.30 (24)
R(IndfB/709)	+	−39.06 ± 1.77 (42)
	−	−0.14 ± 1.41 (40)

The table shows mean response indices (*RI*) ± SE to nonanol of recombinant strains for IndfA and IndfB, divided according to their nonanol olfactory phenotype (+ < −20; > −20). Number of strains tested in parentheses.

The frequency with which mutant and wild-type nonanol phenotypes segregated with *e* and *ca* is shown in Table 2. The data for *IndfA* show that this character is located at ~3-85. The data for *IndfB* are less satisfactory, due to the nonreciprocal distribution of the two recombinant classes. Nevertheless, this distribution is not significantly different from the rectangular distribution that would be expected were *IndfB* to have the same location as *IndfA* (χ^2 goodness of fit = 4.03, d.f. = 3, P = NS). Given the uncertainty associated with a localization based on such small sample sizes, however, it is not possible to conclude anything more than that both *IndfA* and *IndfB* are located between *ebony* (3-70.7) and *claret* (3-100.7). *slowpoke (slo)*, a potassium channel mutation with major behavioral effects in larvae, is located at 3-85. However, this mutation presents a strong wild-type nonanol phenotype (*RI* = −63.22 ± 2.49), indicat-

TABLE 2

Frequency of mutant and wild-type nonanol phenotypes

Marker phenotype	Nonanol phenotype	
	Mutant	Wild type
IndfA		
e +	6	6
+ *ca*	12	11
IndfB		
e +	16	17
+ *ca*	4	12

The table shows frequencies of mutant and wild-type nonanol phenotypes in strains recombinant for the *ebony* (*e*) and *claret* (*ca*) markers in IndfA and IndfB recombinant strains. For details of differentiation of phenotypes, see text and Figure 2.

TABLE 3

Mean nonanol response indices for chromosome *3* deletion stocks and one translocation

Chromosome	Other genetic characteristics	Cytology	*RI*
Df(3R)e-N19	*TM2*	93B; 94	−47.29 ± 6.17 (6)
Df(3R)crbS87-4	*ste/TM3*	95E8-F1; 95F15	−31.87 ± 4.09 (12)
Df(3R)crbS87-5	*ste/TM3*	95F7; 96A17-18	6.38 ± 3.66 (14)
Df(3R)XS, Dp(3R)XS	*aso ats pp/TM6B, e Tb ca; y/y + Y*	96A1-7; 96A21-25	−37.59 ± 5.50 (10)
Df(3R)slo8	*Dp(3;3)Su8*	96A2-9;96D2-4	4.14 ± 2.56 (10)
T(Y;3)A117	*TM6*	96A10-17	−41.9 ± 8.36 (10)
Df(3R)XTAI	*th st ri roe p/Dp (3;3) Su M(3) wl3, st e*	96B; 96D (94D; 96E)	−39.71 ± 7.67 (8)
Df(3R)TI-P	*e ca/TM3*	97A; 98A1-2	−35.24 ± 4.66 (11)
Df(3R)L127	*TM6;Dp(3;1)B152*	99B;99E (98E;100F)	−63.99 ± 2.33 (4)

RI values are means ± SE, with number of observations in parentheses.

ing that *slo-* is not identical to either of the known *Indf* mutations.

The role of the rest of the chromosome in determining the Indf nonanol phenotype was tested by examining the effect of the different morphological markers within a given Indf phenotype class (mutant or wild type). For *IndfA* significant effects were found for *scarlet* (3-44), which, when wild type (*i.e.,* coming from the *IndfA* chromosome) tended to increase the repulsion ($RI = -41.80 \pm 3.73$ and -31.41 ± 1.50, respectively; $t = 2.51$, d.f. = 23, $P < 0.02$). No significant effect was found for *IndfB*.

Deletion mapping: The dominance of *IndfB* would normally render deletion mapping uninformative because deletion/mutation heterozygotes would tend to be indistinguishable from wild-type/mutation heterozygotes. However, the mutant phenotype can be considered to represent the absence of a response, perhaps indicating that the mutation is an amorph and thus providing a rationale for testing deletions. If the wild-type gene were to be haplo-insufficient, deletion/wild type might give a mutant phenotype. Eight deletion stocks and one translocation were tested covering the region 93B-99E (*ebony* is located in 93D2-6, *claret* in 99B5-9) (Table 3). Two deletion stocks showed a mutant nonanol phenotype; all the others showed a wild-type repulsion. This result is clearly the effect of the deletions: *Df(3R)crbS87-4* and *Df(3R)crbSB7-5* produced different nonanol phenotypes, despite sharing the rest of their genome, including their balancer chromosome. The character coding for the wild-type nonanol phenotype is thus a haplo-insufficiency. Figure 3 shows that, on the basis of the overlap of the various deletions, at least one of the *Indf* characters is located in polytene band 96A2-7.

Further defining the IndfA and IndfB olfactory phenotypes: IndfA, IndfB and CS larvae were tested for their responses to 14 different alcohols, acids, acetates and methyl esters. Where qualitatively different responses were found between these three strains, four deletion strains (two showing the mutant nonanol phenotype, two showing the wild-type nonanol phenotype), together with F_1 hybrids between CS, IndfA and IndfB, were tested (Table 4). As a further test, eight recombinant strains showing wild-type nonanol phenotypes (four from each recombination experiment) were also observed. Their responses were not significantly different from those for CS (data not shown). Not all statistically significant differences between the olfactory responses of IndfA, IndfB and CS larvae were tested. For example, significant differences were found for heptanol ($F_{2,23} = 4.75$, $P < 0.02$) and heptanoic acid ($F_{2,23} = 6.35$, $P < 0.001$), both of which induce a strong attraction in all three strains, with relatively minor quantitative differences.

As previously reported, *IndfB* is dominant over CS for

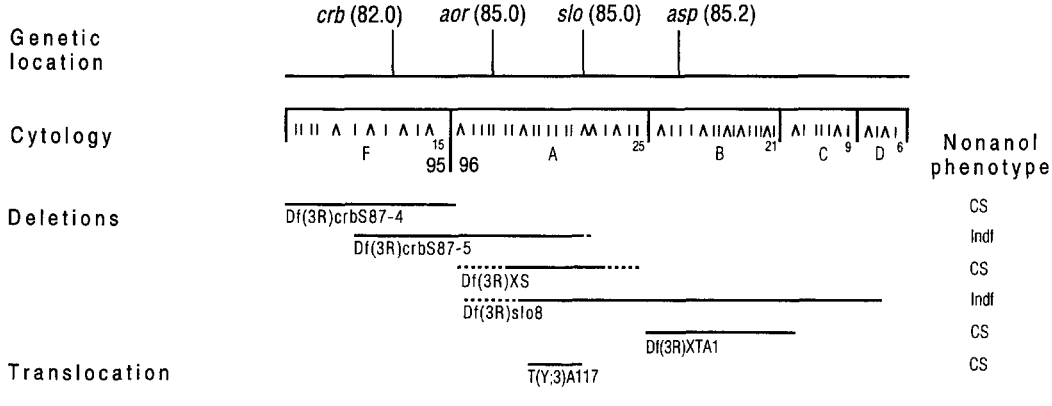
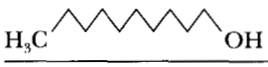
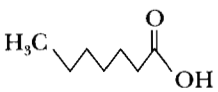
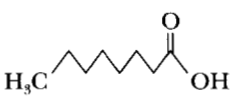
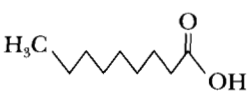
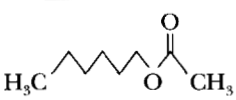
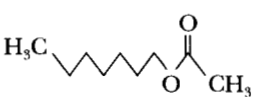
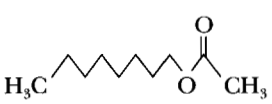
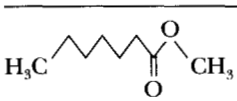
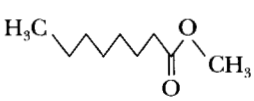
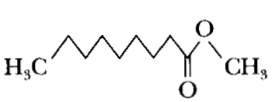
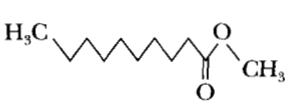


FIGURE 3.—Map of the right arm of chromosome *3* showing genes, cytology and deficiencies. The nonanol olfactory phenotype is described for each deficiency. CS, wild type; Indf, mutant. Data taken from Table 3. The dotted sections of deficiencies *Df(3R)crb-S87-5, Df(3R)XS* and *Df(3R)slo8* indicate that the precise cytological location of the breakpoint is not known. For further details, see Table 3.

TABLE 4

Mean olfactory response indices of IndfA, IndfB, CS and various chromosome *3* deletion stocks and hybrids

Structure	Odor	Carbons	CS	IndfA	IndfB
	Heptanol	C7	41.74 ± 4.69	50.83 ± 3.25	29.82 ± 6.13
	Octanol	C8	8.60 ± 5.98	17.63 ± 4.40	−40.40 ± 4.90
	Nonanol	C9	−41.45 ± 5.05	1.57 ± 3.92	5.26 ± 6.87
	Decanol	C10	−2.47 ± 4.10	−1.70 ± 4.17	−13.64 ± 7.22
	Heptanoic acid	C7	53.54 ± 1.68	74.27 ± 2.43	69.23 ± 3.57
	Octanoic acid	C8	36.34 ± 7.47	53.80 ± 5.56	3.41 ± 3.14
	Nonanoic acid	C9	9.73 ± 5.60	6.24 ± 4.09	1.66 ± 5.06
	Hexyl acetate	C8	−10.87 ± 5.34	7.41 ± 7.60	−0.37 ± 4.41
	Heptyl acetate	C9	−38.45 ± 7.63	−4.10 ± 4.83	−12.16 ± 8.70
	Octyl acetate	C10	−47.12 ± 5.88	−50.72 ± 2.46	−40.59 ± 5.88
	Methyl heptanoate	C8	4.39 ± 6.57	6.94 ± 5.98	−6.36 ± 2.36
	Methyl octanoate	C9	−6.85 ± 9.06	21.77 ± 3.91	20.72 ± 2.49
	Methyl nonanoate	C10	−31.41 ± 6.63	4.11 ± 5.53	−4.54 ± 9.95
	Methyl decanoate	C11	66.24 ± 2.83	62.45 ± 4.00	69.25 ± 4.99

Values are means ± SE. —, not tested.

the nonanol phenotype. *IndfA/CS* hybrid larvae showed a response index that was slightly greater than the empirical cut-off point of the wild-type phenotype (−20), suggesting that *IndfA* may also be dominant. However both strains are recessive for the response to methyl octanoate (Table 4). No complementation was observed, with *IndfA/IndfB* hybrids showing a response that was significantly different from CS (t = 3.5, d.f. = 18, $P < 0.01$), but not significantly different from *IndfA* and *IndfB* ($F_{2,27}$ = 0.73, P = NS), strongly indicating that *IndfA* and *IndfB* are allelic. The fact that the responses of *Df(3R)crbS7-5* and *Df(3R)slo8* larvae, which carry a deletion plus one wild-type copy of *Indf*, showed a response that was not significantly different from wild-type larvae ($F_{2,60}$ = 1.70, P = NS) reinforces the interpretation that the *Indf* wild-type allele is dominant for

Df(3R) crbS7-5	*Df(3R) slo*^{8}	*DF(3R) crbS7-4*	*Df(3R) XS*	CS/IndfA	CS/IndfB	IndfA/IndfB
—	—	—	—	—	—	—
−21.51 ± 5.93	−32.77 ± 3.79	−2.44 ± 4.78	5.25 ± 7.81	−4.33 ± 3.86	−28.65 ± 3.46	−55.55 ± 3.56
5.60 ± 3.73	4.14 ± 2.56	−31.87 ± 4.09	−37.59 ± 5.50	−19.29 ± 2.22	−8.58 ± 4.96	−2.94 ± 7.16
—	—	—	—	—	—	—
—	—	—	—	—	—	—
−10.92 ± 4.19	15.66 ± 3.92	35.94 ± 3.90	29.35 ± 6.78	22.53 ± 2.84	−8.33 ± 2.57	−1.49 ± 6.51
—	—	—	—	—	—	—
—	—	—	—	—	—	—
−7.27 ± 5.30	−0.03 ± 5.49	−20.58 ± 4.22	−39.53 ± 8.28	−17.97 ± 3.98	1.91 ± 3.05	6.59 ± 3.87
—	—	—	—	—	—	—
—	—	—	—	—	—	—
8.58 ± 5.10	6.31 ± 3.68	2.89 ± 4.41	5.40 ± 6.63	−12.92 ± 7.45	−12.38 ± 5.74	31.03 ± 5.15
8.54 ± 4.23	−5.02 ± 2.26	−20.38 ± 3.74	−32.16 ± 4.61	0.40 ± 4.12	5.50 ± 5.21	9.75 ± 5.87
—	—	—	—	—	—	—

the methyl octanoate phenotype and suggests that only one copy is required to express the wild-type phenotype (indifference).

IndfB shows a more extreme phenotypic defect than *IndfA*. The two mutants both failed to respond to nonanol, heptyl acetate and methyl nonanoate, all of which induced repulsion in CS larvae. They were both slightly attracted to methyl octanoate, whilst CS larvae did not respond. With the doses tested here, significant differences were found between IndfA and IndfB strains for octanol ($F_{1,19} = 66.02$, $P = 0.0001$) and octanoic acid ($F_{1,15} = 63.58$, $P < 0.001$). *IndfA* larvae gave a wild-type response to octanol (very slight attraction) and octanoic acid (attraction), while *IndfB* larvae were repulsed by octanol and did not respond to octanoic acid.

In general, *IndfB* produced the same behavioral responses as the appropriate deletion stocks. An ANOVA on the responses of *IndfB, Df(3R)crbS7-5* and *Df(3R)slo8*

larvae to the odors for which all three strains were tested (with the exception of methyl octanoate) revealed no overall significant difference between the strains ($F_{2,131}$ = 1.47, P = NS) with a slightly significant interaction ($F_{8,131}$ = 3.01, $P < 0.05$). This result was due to the significant difference found for the response to octanoic acid ($F_{2,29}$ = 11.96, $P < 0.001$), for which *Df(3R)slo8* showed a slight attraction (*RI* = 15.66 ± 3.92), *Df(3R)crbS7-5* a slight repulsion (*RI* = −10.92 ± 4.19) and *IndfB* no response (*RI* = 3.41 ± 3.14). However, all three responses can be considered as qualitatively similar when compared with the clear attraction shown by CS (*RI* = 36.34 ± 7.47) and *IndfA* (*RI* = 53.80 ± 5.56).

For each of the four classes of odors tested, either IndfA or IndfB larvae showed responses that were qualitatively different from those of CS larvae. The odors involved were octanol (C8) and nonanol (C9), octanoic acid (C9), heptyl acetate (C9) and methyl octanoate (C9) and methyl nonanoate (C10). For each class of odor, responses of Indf larvae to odors with one carbon more or less were normal (Table 4). With the exception of methyl octanoate, which shows a recessive phenotype, these differences were also shown by *Df(3R)crbS7-5* and *Df(3R)slo8* larvae, and not by *Df(3R)crbS7-4* and *Df(3R)XS* larvae, indicating that all five aberrant olfactory phenotypes are localized to 96A2-7 and are almost certainly due to the activity of *Indf*.

No significant differences were observed for the responses of CS, *Df(3R)crbS7-4* and *Df(3R)XS* larvae to octanol, nonanol and octanoic acid. Significant differences were found for the responses to heptyl acetate ($F_{2,20}$ = 6.49, P = 0.008) and methyl nonanoate ($F_{2,22}$ = 3.94, P = 0.036). However, these reflect essentially quantitative differences in the strength of repulsion induced by these two odors in these strains.

DISCUSSION

The *Indifferent* mutations were isolated using an EMS screen for aberrant larval olfactory responses to nonanol. The behavioral defect was initially thought to be limited to nonanol (Cobb *et al.* 1992). Subsequent experiments revealed that the mutants also failed to respond to heptyl acetate (Cobb and Dannet 1994). The central finding of this study is that two deletion stocks consistently showed the same mutant olfactory phenotype as larvae from both Indf strains when tested with five different but closely related odors. Two other deletion stocks consistently showed wild-type responses to these odors. Two deletion stocks (*Df(3R)crbS7-5* and *Df(3R)crbS7-4)* differ only for the deleted region of the third chromosome, but nevertheless gave qualitatively different olfactory responses, indicating that only genes in this region are involved. These results localize *IndfA* and *IndfB* to a small cytological region (96A2-7) and indicate that the behavioral lesion is more pleiotropic than previously thought.

IndfA was initially described as being semidominant, *IndfB* as being dominant (Cobb *et al.* 1992). It was not possible to determine whether *IndfA* and *IndfB* were allelic, because complementation tests are uninformative when carried out with a dominant character. The results presented here show that the dominance of *IndfA* and *IndfB* depends upon the olfactory phenotype being measured. When tested with methyl octanoate *IndfA* and *IndfB* appeared to be recessive; *IndfA/IndfB* hybrids showed no complementation when tested with this odor, strongly suggesting that *IndfA* and *IndfB* are alleles. Changing patterns of dominance/recessivity for a given allele depending on the phenotype studied are well known in Drosophila (Muller 1932), but this example is relatively unusual in that the phenotypes involved are very similar (olfactory responses to two slightly different odors).

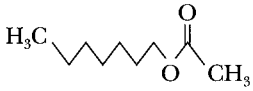
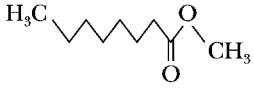
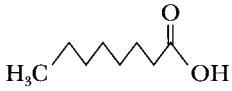
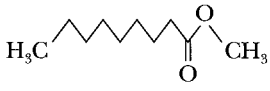
The fact that deletion stocks can show a mutant nonanol phenotype indicates that *Indf* is a haplo-insufficiency. Two functional copies are thus required for the wild-type nonanol phenotype; this explains why the mutant strains are dominant for their nonanol phenotype. *IndfB* larvae generally showed responses that were not significantly different from those of *Df(3R)crbS7-5* and *Df(3R)slo8* deletion stocks (Table 4), perhaps indicating that the IndfB protein is the equivalent of a deletion, *i.e.*, is non-functional. However, the fact that *IndfB* larvae responded to methyl octanoate while both *Df(3R)crbS7-5* and *Df(3R)slo8* larvae, which carry deletion/*Indf*$^+$ showed a (phenotypically wild type) indifference suggests that this interpretation, while it may be partially correct, is not sufficient to explain the observed results.

One test that was not carried out here was to measure the responses of *Indf/deletion* to methyl octanoate (for which *IndfA* and *IndfB* are recessive). This would test the formal possibility that the *Indf* mutations identify one gene that affects the response to nonanol and the other odors tested here, while the 96A deficiencies uncover a second gene that produces a virtually identical phenotype. One way of testing this hypothesis while avoiding the problem of potentially biased results due to testing larvae and measuring the phenotype on the basis of eclosed adults that can introduce differential mortality effects (see MATERIALS AND METHODS) would be to make new deletion stocks over *TM6B,* which carries the larval marker *Tubby* (see Table 3). This would enable *Indf/deletion* larvae to be observed directly. However, even if this test were to support the "two genes" hypothesis, this would still indicate that chromosomal region 96A2-7 contains a gene involved in olfaction that is worthy of further investigation.

IndfA larvae showed wild-type responses to octanoic acid (both C8), while IndfB, *Df(3R)crbS7-5* and *Df(3R)slo8* larvae showed mutant responses (Table 4). These data suggest that the IndfA protein is at least partly functional; this could explain the borderline dominance of the *IndfA* character

TABLE 5

Summary of olfactory responses of larvae of CS, IndfA, and IndfB strains to six odors

Structure	Odor	Carbons	CS	IndfA	IndfB	No. of Copies
	Nonanol	C9	−−	0	0	2
	Heptyl acetate	C9	−−	0	0	2
	Methyl nonanoate	C10	−−	0	0	2
	Octanol	C8	0	+	−−	2
	Octanoic acid	C8	++	++	0	2
	Methyl octanoate	C9	0	+	+	1

0, indifference ($-15 < RI < 15$); +, slight attraction ($15 < RI < 30$); ++, attraction ($30 < RI$); −−, repulsion ($-30 > RI$). No. of copies, number of copies of wild-type allele required for wild-type response.

over CS when tested with nonanol, although the presence of modifying factor(s) on the *IndfA* chromosome in the vicinity of *scarlet* may also play a role.

These results imply that *Indf* is probably not a structural gene, coding for, say, a receptor. The quantity of the Indf protein is apparently important for the olfactory phenotype, the protein presumably being directly or indirectly involved in the detection of an "odor primitive" (some fundamental physico-chemical aspect of the molecule) related to odor molecules in the range C8-C10. As the summary in Table 5 shows, however, the exact nature of this "odor primitive" is difficult to determine: the gene does not appear to be involved in the processing of number of carbons (methyl nonanoate (C10) elicits a mutant response, decanol (C10) does not), nor chain length (nonanol (chain-length = 9) elicits a mutant response, methyl decanoate (chain-length = 9) does not), nor double-bond position (octanoic acid (Δ 8) elicits a mutant response, hexyl acetate (Δ 8) does not).

Furthermore, how can these data be integrated with the larva's relatively simple olfactory system? "Odor primitives" by definition imply the existence of integrative structures in order for the organism to assemble a sensory image from various "primitives". In the adult fly, this kind of function is carried out by the glomeruli (STOCKER 1994). No such structures exist in the larva, which only possess 21 olfactory neurons (SINGH and SINGH 1984). This may either indicate that Drosophila larval olfactory neurons produce signals that undergo no further processing but which directly represent the odor(s) to which the receptors are tuned, or that some simple processing does take place, probably in the brain, to which the olfactory nerve projects (STOCKER 1994).

Although the exact role of the Indf protein will require further investigation, the data presented in Table 5 do nevertheless give some insight into olfactory coding in these insects. CS larvae were repulsed by nonanol (C9), heptyl acetate (C9) and methyl nonanoate (C10), while *IndfA, IndfB, Df(3R)crbS7-5* and *Df(3R)slo8* larvae were indifferent to these three substances. This suggests either that these three substances are sensory equivalents for Drosophila larvae or that they share coding structures that are affected by the *Indf* mutation or both. The detection or processing of these substances may constitute the primary function of *Indf*. Both IndfA and IndfB strains were attracted by methyl octanoate (C9), while wild-type larvae and all deletion stocks were indifferent. This suggests that this substance is not a sensory equivalent of nonanol, heptyl acetate and methyl nonanoate, but that it shares some common coding structure. In order to resolve these problems, electrophysiological studies of mutant and deletion stocks will be necessary. This may be possible on adult strains: COBB *et al.* (1992) reported that *Indf* mutants showed aberrant

responses to nonanol as adults, indicating that common genes may be involved in olfactory phenotypes in larvae and adults.

A major challenge for future studies will be to disentangle dose-response effects from absolute anosmias. For example, IndfB larvae gave a strong, mutant, repulsive response to octanol at the volumes tested here. However, when tested at higher volumes, IndfB larvae failed to respond and were not significantly different from wild type (COBB *et al.* 1992). This indicates that the *IndfB* mutation apparently involves some sensitive threshold detection.

A number of genes are known from the 96A region of the chromosome that are potential candidates for the *Indf* gene, all of which will require investigation. Genes coding for three of the five known subunits of nicotinic acetylcholine receptors in Drosophila are found in the 96A region (NEDLINKA-CHITTKA and GUNDELFINGER 1992). A haplo-insufficient morphological mutation, *Minute(3)96A,* is also located in this region (LINDSAY and ZIMM 1992). Although none of the strains studied here were observed to have the short bristles typical of this mutation, the fact that both *Indf* and *M(3)96A* are haplo-insufficiencies clearly makes this gene worth investigating. A number of isoforms of protein phosphatase 1 are known to be coded for by genes in this region (CHEN *et al.* 1992) as is *abdominal one reduced,* an adult phenotype that shows a reduction in the first abdominal stergite (GONZALEZ *et al.* 1989). It is also interesting to note that a newly discovered gene affecting larval foraging behavior, *Chaser,* has been localized to 95F7-96A1 (PEREIRA *et al.* 1995), perhaps indicating the presence of a cluster of genes involved in larval behavior in this region.

Previous studies of olfactory mutants in Drosophila have generally investigated the responses of adult flies to a few odors carrying different chemical functions (see CARLSON 1996 for a review). In this study, larval olfactory responses to 14 structurally related odors were studied. The behavior of the *Indf* mutants reveals that three of these odors—nonanol (C9), heptyl acetate (C9) and methyl nonanoate (C10)—share common coding structures and thus gene(s) and may be the "target" of the *Indifferent* gene. The phenotypic specificity of this mutation, the strength of the mutant phenotype (anosmia) and the relatively simple neural network within which the gene functions indicate that this character has an important potential for furthering our understanding of the relationship between genes and olfaction. Further investigation of the nature of the $Indf^-$ lesion(s) and the function of the Indf protein should reveal not only how the Drosophila larval olfactory system functions, but may also provide an insight into the principles of olfactory coding that could apply to other organisms.

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