

abolished the activation of both MEKK and B-Raf by EGF, NGF, and TPA (Fig. 6). These results demonstrate that PKA activation inhibits growth factor stimulation of the 98-kD MEKK, suggesting the existence of a common regulatory control point for PKA action that lies between or downstream of Ras and upstream or at the level of each of the three kinases.

Our results show that MEKK is regulated by growth factors in a Ras-dependent manner in PC12 cells. Thus, Ras serves as a common control point for the regulation of both MEKK and Raf protein kinases (Fig. 7). Insulin-stimulated activation of MAPK and 90-kD ribosomal protein S6 kinase (RSK) is blocked by expression of N¹⁷Ras, but not by expression of dominant negative Raf mutants in 3T3 L1 cells (27), suggesting that Raf-independent pathways exist downstream of Ras. Treatment of adipocytes with insulin results in rapid transient activation of a MEKK of approximately 56 kD that is distinct from Raf-1 (28). These data support a role for Ras-dependent MEKKs in growth factor- and PKC-mediated signaling pathways. Unlike overexpression of Raf, overexpression of wild-type or constitutively active MEKK mutants is not transforming in fibroblasts, and stable expression appears to be growth inhibitory or lethal (17). This indicates that MEKK function must diverge from that of the Raf family members.

Parallel MAPK pathways regulate diverse cellular functions including mating, osmotic regulation, and cell wall biosynthesis in yeast (14, 15). These MAPK pathways function largely independently of one another, suggesting that there is little cross-over in the regulation of substrates by the respective kinases in each pathway within the cell. Similar parallel pathways may exist in mammalian cells. Although members of the Raf family have been detected only in higher eukaryotes and are not present in yeast, MEKKs are expressed in both yeast and metazoan organisms (14, 15). Researchers are identifying a growing number of MAPKs (29, 30), MEKs (5-7, 31), and MEKKs (12, 16) that are expressed in mammalian cells. When Raf kinases and MEKKs are properly assembled in cells with their constituent MEKs and MAPKs, they will predictably regulate different cellular functions. A key to defining these different functions will involve determining which MEKs are preferentially regulated by Raf kinases compared with MEKKs in different cell types and tissues.

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Genetics of a Pheromonal Difference Contributing to Reproductive Isolation in *Drosophila*

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Although sexual isolation is one of the most important causes of speciation, its genetic basis is largely unknown. Here evidence is presented that suggests that sexual isolation between two closely related species of *Drosophila* is largely caused by differences in female cuticular hydrocarbons. This difference maps to only one of the three major chromosomes, implying that reproductive isolation might have a fairly simple genetic basis. The effect of the hydrocarbons on courtship may help explain the ubiquitous asymmetry of sexual isolation between many pairs of *Drosophila* species.

One of the major unsolved questions of evolution is the genetic basis of speciation. Determination of the number, location, and effects of genes producing reproductive isolation bears critically on different theories of speciation and on the controversial idea that important evolutionary change may involve relatively few genes (1-3). Moreover, the mapping and localization of such loci may help reconstruct the origin of species on a gene-by-gene basis, and the subsequent molecular isolation of such genes might help

determine their normal function within species. A recent interest in the genetics of postzygotic reproductive isolation (hybrid sterility and inviability) has yielded several theories of speciation (4). However, prezygotic isolation, especially mating discrimination, may be the primary cause of speciation in many animal taxa (5). There have been relatively few genetic analyses of this trait, although examples exist of both major gene and polygenic determination (1). This rarity reflects the difficulty of behavioral genetic work in many species, as well as the notorious lability of sexual behavior in the laboratory. Here we present evidence that an easily measured morphological character, the cuticular hydrocarbon profile, is a

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cause of sexual isolation in one *Drosophila* group. We also demonstrate that the difference between two closely related species maps to only a single chromosome, and we reconstruct a plausible evolutionary scenario for the origin of sexual isolation in this group.

Sexual isolation in insects is frequently mediated by volatile or contact pheromones, the latter often being long-chain hydrocarbons located on the cuticle surface (6, 7). In some *Drosophila* species, epicuticular hydrocarbons induce male courtship, probably by stimulating chemoreceptors on the male foreleg or proboscis (8–10). Closely related but sexually isolated species often show large differences in the composition and quantity of these compounds (9, 11), but their role in sexual isolation, as well as the genetic basis of species differences, is unclear.

One of the best studied *Drosophila* clades includes the four sibling species *D. melanogaster* and *D. simulans* (cosmopolitan human commensals), and *D. sechellia* and *D. mauritiana* (endemics of Indian Ocean islands). *Drosophila melanogaster* is an outgroup to the other three species, whose phylogeny is not yet resolved (12). Like many *Drosophila* species, these four show asymmetrical sexual isolation: in any hybridization, matings in one reciprocal cross occur far more readily than in the other (13, 14, 15). These four species fall into two groups with respect to cuticular hydrocarbons. In the first group, *D. simulans* and *D. mauritiana* are sexually monomorphic: both males and females have *cis* 7-tricosene (7-T) as the predominant cuticular compound. In the second group, *D. melanogaster* and *D. sechellia* are sexually dimorphic: males have high concentrations of 7-T (as well as 6-tricosene in *D. sechellia*), and females have almost no 7-T but have very large amounts of *cis cis* 7,11-heptacosadiene (7,11-HD), a compound completely absent in *D. simulans* and *D. mauritiana* (Table 1) (9, 10, 16).

In this latter group, males of species sexually monomorphic for 7-T will not court females of sexually dimorphic species, whereas males of the sexually dimorphic species will court females of all four species (15). It has been suggested that interspecific differences in cuticular hydrocarbons could explain much of the sexual isolation, as well as its asymmetry, if a male will court a female only when she carries either his own predominant hydrocarbon or that of his conspecific females (15). The following experiments support this hypothesis, providing evidence that much of the sexual isolation in this group is based on epicuticular hydrocarbons.

Our behavioral observations (Table 2) confirm previous reports that *D. simulans* males almost never court or copulate with *D. sechellia* females and court *F*₁ hybrid

females at intermediate levels (15, 17). These males also vigorously court dead conspecific females that have been flash-frozen, but they largely ignore dead *D. sechellia* females and *F*₁ hybrids (Table 2). The similarity in mate discrimination whether or not females are alive implies that female behavior is not a critical factor in the sexual isolation and implicates a chemical or morphological inducer of courtship. The effect on courtship of the *F*₁ females being dead, however, suggests that female behavior may facilitate courtship of this genotype.

Evidence that male sexual behavior is largely induced or inhibited by epicuticular hydrocarbons comes from our discovery that these hydrocarbons are transferred between females of different species when they are crowded together (Table 1). The effect is reciprocal between *D. sechellia* and *D. simulans* and results in a female acquiring nearly half the amount of the predominant hydrocarbon of the other species. *Drosophila simulans* males court these perfumed females very differently from control females: They almost completely ignore conspecific females carrying 7,11-HD acquired from *D. sechellia* but readily court the controls (Table 2). This

difference in courtship is observed even when the females are dead (Table 2), so the failure of males to court must not be caused by a behavioral effect of heterospecific crowding, but by some substance transferred between females of different species.

Conversely, live or dead *D. sechellia* females that acquire high amounts of 7-T from *D. simulans* are more attractive to *D. simulans* males than the nonperfumed control females (Table 2), although the increase in attraction is not as pronounced as the inhibition accompanying transfer of 7,11-HD. We suspect that the transferred cuticular hydrocarbons cause these changes in female attractiveness, although we cannot rule out the possibility that substances other than 7-T and 7,11-HD are responsible, or that, as in other insects, males are attracted by complex mixtures of compounds rather than single compounds (18, 19).

However, further support that these hydrocarbons are involved is that their transfer also explains the changes in sexual attractiveness of *D. simulans* females previously crowded with females of the two remaining species. Crowding with *D. mauritiana* females, which also have high amounts of 7-T, does not reduce the

Table 1. Main cuticular hydrocarbons of pure species, hybrids, and individuals in pheromone transfer experiments. These compounds were extracted and analyzed with a modification of a described procedure (23, 26). The abbreviation 7-T is *cis* 7-tricosene, 7-P is *cis* 7-pentacosene, and 7,11-HD is *cis cis*-7,11-heptacosadiene. Standard errors are those of single-fly quantities among the replicate samples. All *F*₁ hybrids had *D. simulans* mothers. The eight backcross genotypes are designated by the markers contributed by the *F*₁ mother; the other haploid genome is pure *D. sechellia*. For pheromone transfer, we confined flies in approximately 6 cm³ of space in a vial containing food. Eight newly eclosed females of one species, identified by wing clipping, were crowded with 45 females of either another species (experimental) or their own species (control). The species in higher number is shown in parentheses. After 4 days, wing-clipped flies were separated and used for gas chromatography or the behavioral observations described in Table 2. For both 7-T and 7,11-HD, significant amounts of heterospecific pheromones were transferred by crowding compared with pure species (one-tailed *t* tests give *P* < 0.004 for all four comparisons).

Species and strain	Female hydrocarbons (ng/fly) (SE)			Number of samples
	7-T	7-P	7,11-HD	
	<i>D. sechellia</i>			
1	11.3 (0.6)	59.7 (4.8)	319.9 (24.8)	17
<i>zn; cn</i>	8.9 (0.7)	38.1 (2.5)	406.3 (27.8)	16
	<i>D. simulans</i>			
Florida	627.4 (44.8)	59.2 (7.3)	0	17
H/+	466.5 (27.1)	51.9 (3.2)	0	17
	<i>Hybrids</i>			
<i>F</i> ₁ (Florida × 1)	421.3 (20.5)	248.9 (13.2)	110.2 (9.6)	22
<i>F</i> ₁ (<i>zn; cn</i> × H/+)	241.5 (10.9)	121.3 (7.2)	60.4 (5.8)	15
	<i>Backcross</i> [<i>F</i> ₁ (<i>zn; cn</i> × H/+) × <i>zn; cn</i>]			
<i>zn; cn; +</i>	39.1 (9.7)	79.6 (7.4)	262.4 (23.4)	21
<i>zn; cn; H</i>	126.0 (13.4)	76.9 (8.6)	148.4 (15.8)	21
<i>zn; +; +</i>	38.6 (6.0)	79.7 (11.7)	223.5 (22.5)	21
<i>zn; +; H</i>	144.8 (11.1)	84.8 (5.0)	123.8 (19.4)	21
<i>+; cn; +</i>	27.4 (6.6)	89.5 (8.9)	228.9 (27.6)	21
<i>+; cn; H</i>	133.3 (9.2)	88.1 (8.6)	144.0 (16.8)	21
<i>+; +; +</i>	45.9 (12.3)	86.9 (8.0)	245.0 (29.7)	21
<i>+; +; H</i>	151.3 (12.5)	120.8 (10.4)	136.6 (15.5)	21
	<i>Hydrocarbon transfer</i>			
<i>D. simulans (simulans)</i>	427.2 (38.4)	48.8 (1.9)	0	4
<i>D. simulans (sechellia)</i>	228.4 (19.6)	42.4 (2.8)	85.2 (9.7)	4
<i>D. sechellia (simulans)</i>	201.3 (30.0)	46.4 (4.7)	103.6 (10.2)	4
<i>D. sechellia (sechellia)</i>	8.5 (1.4)	51.2 (2.5)	186.2 (11.8)	4

sexual attractiveness of *D. simulans* females (Table 2), but the reduction is pronounced when they are crowded with *D. melanogaster* females, who have high amounts of 7,11-HD (Table 2).

We investigated the genetic basis of the difference in major hydrocarbons between *D. simulans* and *D. sechellia*. The F₁ hybrid females (Table 1) have intermediate quantities of both hydrocarbons and an elevated amount of 7-pentacosene (7-P), also posited to induce male courtship in *D. simulans* (15). Genetic analysis was performed by crossing *D. sechellia* males homozygous for recessive eye color markers on the X [*xinfandel* (*xn*), 1–26] and second chromosome [*cinnabar* (*cn*), 2–63] to *D. simulans* females heterozygous for the dominant third-chromosome allele *Hairless* (*H*, 3–61) and backcrossing the F₁ *Hairless*/+ females to *D. sechellia* *xn; cn* males. This backcross yields eight distinguishable classes of progeny carrying all possible combinations of the markers. Each marker is on average associated with 85 centimorgans of conspecific genome, nearly the entire chromosome (20). Table 1 gives the hydrocarbon composition of females of each genotype, and Fig. 1 the ratio

of 7,11-HD to 7-T. This ratio is determined almost entirely by the species origin of the third chromosome. Analysis of variance shows that only the *Hairless* marker is associated with the quantity of both major hydrocarbons. *Hairless* flies have significantly higher amounts of 7-T and lower amounts of 7,11-HD than flies carrying the alternative wild-type allele [7-T: $F(1,160) = 184.1, P < 0.0001$; 7,11-HD: $F(1,160) = 61, P < 0.0001$]. No interactions among markers were significant, implying a lack of interchromosomal epistasis. Elevated amounts of 7-P were associated with only the X chromosome [$F(1,160) = 6.6, P = 0.01$]; again, no other marker or interaction was significant. The involvement of only one chromosome in the major hydrocarbon differences may mean that these differences reside at a single locus, although it is possible that they derive from several genes on the third chromosome. This possibility can eventually be tested with the panoply of molecular tools available in *Drosophila*. It seems likely, however, that the character difference has a simple genetic basis and does not derive from evolutionary changes spread throughout the genome.

For *D. simulans* males, 7,11-HD appears to be a more potent inhibitor of courtship than 7-T is an attractant. If this is true, an evolutionary transition from the sexually dimorphic to the sexually monomorphic hydrocarbon state may be easier than the reverse transition, because the former can be accomplished without loss of male courtship. There is evidence that evolutionary changes in moth pheromone and receptor systems began with the production of a new female signal, followed by changes in male preference (21). Such a transition apparently occurred in this *Drosophila* clade, because the outgroup species *D. melanogaster* is, like *D. sechellia*, sexually dimorphic for hydrocarbons. *D. sechellia* may then have retained the ancestral character, leading to the prediction that the major hydrocarbon difference between *D. simulans* and *D. melanogaster* would also map to the third chromosome. If such sexually dimorphic hydrocarbon effects occur widely, they might also help explain the well-known but poorly understood asymmetry of sexual isolation between many pairs of *Drosophila* species (22).

Our genetic analysis, combined with changes in mating behavior that accompany hydrocarbon transfer, suggest that sexual isolation among species may sometimes have a simple genetic basis. This is supported by two studies of single-gene changes that cause pheromonal hydrocarbon differences in Lepidoptera (6, 19). The change in female pheromones must, of course, be accompanied by evolutionary changes in male perception or choice, which, though genetically analyzed in the lepidopteran studies, have not been examined in this group of *Drosophila*. Some African populations of *D. simulans* have reduced amounts of 7-T and high amounts of 7-P, a polymorphism mapping

Table 2. Tests of sexual isolation with *D. simulans* males confined with various females. Eight treatments are shown, each comparing the courtship behavior of *D. simulans* males with two types of females. Each vial contained four *D. simulans* males and two females. Dead females were killed by instant immersion in liquid nitrogen and placed 1 cm apart at the edge of the medium (such freezing removes less than 5% of the total cuticular hydrocarbons). The pair or trio of vials were watched for 30 min by an observer who scored courtship behavior without knowing the identities of the females (27). Data in the table are the mean number of behaviors per vial averaged over all replicate vials. We compared the frequencies of behaviors between control and experimental groups by applying the nonparametric Mann-Whitney *U* tests to the two compared groups of replicates; thus, each comparison involved two groups of either 20, 30, or 40 integers (28). When three groups were watched simultaneously (treatments 1 and 2), two of them were compared with the pure *D. simulans* females. Probabilities are from the nonparametric test, but statistical significance does not change with the use of parametric *t* tests. Co-reared flies were treated as described in Table 1; the species in parentheses is the species with which tested flies were crowded. Species names are represented by their first three letters. Dashes indicate that no copulation occurred.

Treatment	Female type	Females live (L) or dead (D)	Replicates	Mean acts per vial		
				Courtship episodes (SE)	Attempted copulations (SE)	Copulations (SE)
<i>Pure species</i>						
1	<i>sim</i>	L	20	7.25 (0.77)	6.10 (0.86)	1.80 (0.09)
	F ₁			6.25 (1.83)	2.75 (0.97)***	0.50 (0.17)***
	<i>sec</i>			0.15 (0.11)***	0***	0***
2	<i>sim</i>	D	20	13.95 (2.74)	9.80 (1.98)	–
	F ₁			0.95 (0.34)***	0 (0)***	–
	<i>sec</i>			0.50 (0.24)***	0 (0)***	–
<i>Hydrocarbon transfer</i>						
3	<i>sim (sim)</i>	L	20	13.40 (2.96)	12.80 (2.63)	1.95 (0.09)
	<i>sim (sec)</i>			1.10 (0.32)***	0.50 (0.21)***	0.60 (0.17)***
4	<i>sim (sim)</i>	D	20	11.4 (2.27)	5.45 (1.54)	–
	<i>sim (sec)</i>			0.85 (0.53)***	0 (0)***	–
5	<i>sec (sec)</i>	L	30	0.17 (0.07)	0 (0)	0
	<i>sec (sim)</i>			2.59 (0.47)**	0.33 (0.18)*	0
6	<i>sec (sec)</i>	D	40	0.17 (0.09)	0 (0)	–
	<i>sec (sim)</i>			0.51 (0.16)*	0.05 (0.04)	–
7	<i>sim (sim)</i>	L	20	10.15 (2.46)	5.85 (1.43)	1.95 (0.05)
	<i>sim (mau)</i>			7.45 (1.61)	5.05 (0.88)	1.95 (0.05)
8	<i>sim (sim)</i>	L	20	8.65 (1.41)	8.40 (2.40)	2.00 (0)
	<i>sim (mel)</i>			0.35 (0.17)***	0.25 (0.20)***	0.15 (0.82)***

P* < 0.05. *P* < 0.01. ****P* ≤ 0.001.

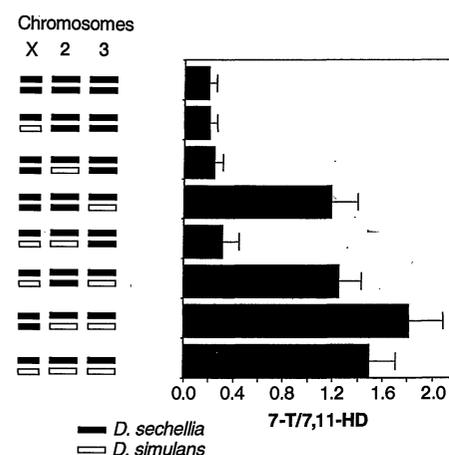


Fig. 1. Ratio of 7-tricosene to 7, 11-heptacosadiene in the eight backcross genotypes described in Table 1. Shading of chromosomes show the species origin of the source of marker genes. Ratios for each genotype are the means of 21 samples of two flies each. Error bars show one standard error of this ratio.

to a single second-chromosome gene (23), and mutagenesis has produced other *D. simulans* alleles with large effects on hydrocarbon profiles (24). [Such polymorphism may explain the observation in *Drosophila* of intraspecific genetic variation in the propensity to initiate courtship with other species (14).] All this suggests that the evolution of sexual isolation in this group may sometimes be caused by changes in only a few genes (2). This in turn militates against the evolution of sexual isolation by long-term runaway processes, which might produce polygenic sexual isolation (1) and, in conjunction with the phylogenetic data, suggests scenarios beginning with the evolution of female traits or mating preferences. It is important to note, however, that other cues besides pheromones, such as auditory and visual signals, almost certainly play a role in sexual isolation among *Drosophila* species.

Finally, the mapping of the hydrocarbon differences to a chromosome does not support recent ideas that speciation is often caused by the acquisition of symbionts (25) but adds instead to the considerable data implicating changes in nuclear genes. *Drosophila* is a valuable group for isolating and characterizing these genes.

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26. The dried extract (23) was redissolved in 5 μ l of n-hexane, 3 μ l of which was injected into a HP 5890 Series II gas chromatograph connected to a HP 3396 Series II integrator. The temperature was increased from 210° to 250°C at 3°C/min, each run lasting 20 min. The split ratio was 50:1. Hydrocarbon peaks were identified by comigration with known standards and analysis on a VG Analytical 70-70 mass spectrometer. Depending on the sample, we detected between 15 and 25 peaks, with 7-T constituting ~48% of the total sample in *D. simulans* and 7,11-HD constituting ~25% of the total sample in *D. sechellia*. These proportions are similar to those obtained in a previous study (9). Absolute quantities of hydrocarbons were estimated by comparison of peak areas with those of an internal *N*-hexacosane standard added to each sample. All samples contained extract from either two flies (eight backcross genotypes) or four flies (all other samples).
27. Courtships were defined as any episode which be-

gan with a male orienting toward the female and vibrating his wings (10); such episodes ended when the male no longer oriented toward the female. Attempted copulations were defined as any instance in which the male curled his abdomen ventrally and attempted to mount the female. Copulations with dead females were never seen.

28. We also measured courtship duration in a separate experiment with single *D. simulans* males confined with single females from the two groups in treatment 3. We watched 45 pairs of vials for 10 min each; scoring was again blind. Pure *D. simulans* females raised under crowded conditions (controls) were courted 72 times with mean courtship length of 1.12 min (SD = 1.66). *D. simulans* females crowded with *D. sechellia* females were courted 26 times with mean courtship length of 0.33 min (SD = 0.47). Therefore, co-rearing with *D. sechellia* females significantly shortened the duration of courtship bouts (Mann-Whitney *U* test, $Z = 3.00$, two-tailed $P = 0.003$), giving further evidence for a transferable substance inhibiting courtship by *D. simulans* males.
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Rapid Induction of Alzheimer A β Amyloid Formation by Zinc

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A β_{1-40} , a major component of Alzheimer's disease cerebral amyloid, is present in the cerebrospinal fluid and remains relatively soluble at high concentrations (less than or equal to 3.7 mM). Thus, physiological factors which induce A β amyloid formation could provide clues to the pathogenesis of the disease. It has been shown that human A β specifically and saturably binds zinc. Here, concentrations of zinc above 300 nM rapidly destabilized human A β_{1-40} solutions, inducing tinctorial amyloid formation. However, rat A β_{1-40} binds zinc less avidly and is immune to these effects, perhaps explaining the scarcity with which these animals form cerebral A β amyloid. These data suggest a role for cerebral zinc metabolism in the neuropathogenesis of Alzheimer's disease.

The role of A β amyloid formation in the pathogenesis of Alzheimer's disease (AD) has been underscored by the discovery of

mutations in the Alzheimer amyloid protein precursor (APP) close to or within the A β domain that are linked to familial AD (1). A β is found as a 40-residue peptide (A β_{1-40}) in cerebrospinal fluid (CSF) but is not found at elevated concentrations in sporadic AD cases (2). Synthetic A β_{1-40} remains soluble in neutral phosphate buffer at concentrations of up to 16 mg/ml (3), indicating that overproduction of soluble A β cannot sufficiently explain A β precipitation. Therefore, biochemical mechanisms that promote A β amyloid formation in sporadic cases may be relevant to the pathogenesis of AD. We have shown that A β specifically and saturably binds Zn, manifesting both high-affinity binding ($K_A = 107$ nM) compatible with normal CSF Zn concentrations, and low-affinity binding ($K_A = 5.2$ μ M) (4). Cerebral Zn homeosta-

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