Scribble Is Essential for Olfactory Behavior in *Drosophila melanogaster*

Indrani Ganguly,*,† Trudy F. C. Mackay*,† and Robert R. H. Anholt*,†,‡,[1]

**The W. M. Keck Center for Behavioral Biology,* †*Department of Genetics and* ‡*Department of Zoology, North Carolina State University, Raleigh, North Carolina 27695*

Manuscript received December 9, 2002
Accepted for publication April 4, 2003

ABSTRACT

The ability to discriminate and respond to chemical signals from the environment is an almost universal prerequisite for survival. Here, we report that the scaffold protein Scribble is essential for odor-guided behavior in Drosophila. Previously, we identified a *P*-element insert line with generalized sexually dimorphic smell impairment, *smi97B*. We found that the transposon in this line is located between the predicted promoter region and the transcription initiation site of *scrib*. A deficiency in this region, *Df(3R)Tl-X*, and two *scrib* null alleles fail to complement the smell-impaired phenotype of *smi97B*. Wild-type behavior is restored by precise excision of the *P* element, *scrib* mRNA levels correspond with mutant and wild-type phenotypes, and introduction of a full-length *scrib* transgene in the *smi97B* mutant rescues the olfactory deficit. Expression of Scrib is widespread in olfactory organs and the central nervous system. Finally, alternative splicing of *scrib* generates transcripts that differ in the number of leucine-rich repeats and PDZ domains.

FOR virtually all animals, behavioral responses to odorants are essential for food localization, avoidance of noxious agents, and selection of reproductive partners. Olfactory behavior is a complex trait, attributable to multiple interacting loci with small and environmentally sensitive effects. While the genetic basis of odorant recognition has been extensively investigated (Fox *et al.* 2001; Mombaerts 2001; Vosshall 2001; Warr *et al.* 2001; Zhang and Firestein 2002), little information is available about the genetic networks that subserve processing of olfactory information and directing appropriate behavioral responses to odorants.

Genetic dissection of complex behavior has traditionally followed one of two approaches: analysis of induced mutations with severe behavioral effects and quantitative genetic studies of naturally occurring behavioral variation (Hirsch 1967; Benzer 1973; Tully 1996; Greenspan 1997; Sokolowski 1998). The major advantage of the mutational approach is the ability to identify genes required for producing behavior. However, screens for severe mutations will not detect loci with pleiotropic effects on behavior at which null mutations are homozygous lethal or loci for which functional redundancy precludes large mutational effects. Combining quantitative genetic with mutational analysis to detect adult viable mutations with subtle behavioral effects in controlled, isogenic genetic backgrounds should facilitate the discovery of such new loci (Nadeau and Frankel 2000; Mackay 2001).

We applied this strategy to identify 14 novel, autosomal, *P*-element-tagged *smell-impaired* (*smi*) loci in *Drosophila melanogaster* (Anholt *et al.* 1996), all of which had subtle but statistically significant reduced avoidance responses to benzaldehyde, a repellent odorant. Interestingly, the mutational effects for 4 of these loci were sexually dimorphic, with larger effects in females than in males. Genes that affect naturally occurring variation in olfactory avoidance response to benzaldehyde also have different effects in males and females (Mackay *et al.* 1996). Variation in sexual dimorphism is thus an integral aspect of the genetic architecture of olfactory behavior, as well as other quantitative traits in Drosophila (Mackay 2001). In keeping with this observation, as much as 60% of the Drosophila transcriptome shows sex-biased expression (Jin *et al.* 2001; this is an upper estimate, as it includes germline transcripts).

Here, we show that *smi97B*, a *P*-element-induced mutation with sex-specific effects on olfactory behavior, is an allele of *scribble* (*scrib*), a pleiotropic gene that encodes 16 leucine-rich repeats (LRR) and four PDZ (*P*SD-95, *D*lg, *Z*O-1) domains and is essential for establishing polarity in epithelial cells during embryonic development (Bilder and Perrimon 2000; Bilder 2001), tumor suppression (Bilder *et al.* 2000; Li *et al.* 2001), synaptogenesis (Mathew *et al.* 2002; Roche *et al.* 2002), and immune responsiveness (Wu *et al.* 2001). We have identified at least seven alternatively spliced *scrib* variants in adults, including one that is more abundant in males and another that is female-specific. These studies contribute to the emerging theme that mutations and naturally occurring alleles affecting behavior are subtle vari-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY167903–AY167906.

[1]*Corresponding author:* W. M. Keck Center for Behavioral Biology, Campus Box 7617, North Carolina State University, Raleigh, NC 27695-7617. E-mail: anholt@ncsu.edu

ants of pleiotropic genes, often with sexually dimorphic phenotypic effects (Bellen and Kiger 1987; Price *et al.* 1998; Shaver *et al.* 1998; Anand *et al.* 2001; Toma *et al.* 2002).

MATERIALS AND METHODS

Fly stocks: The *smi97B* mutant was generated by mobilization of a *P[lArB]* element to cytological location 97B2 in the highly inbred *Samarkand* (*Sam*); ry^{506} strain (Anholt *et al.* 1996). EMS-generated nulls, $scrib^{1}$ and $scrib^{2}$, and *P[lacW]* insertions $scrib^{S042405}$ and $scrib^{j7B3}$ were balanced over *TM3, Sb. Df(3R)Tl-X* and $scrib^{j7B3}$ were obtained from the Bloomington *Drosophila* Stock Center. All other *scrib* stocks, including a transgenic stock containing a full-length *scrib* cDNA clone on the *X* chromosome, were provided by D. Bilder. Stocks used in the derivation of *P[lArB]*-element excision lines of *smi97B* and those used to introgress the transgenic stock into the *smi97B* homozygous background were *Sam1; Sam2; Sb* Δ*2-3/Ubx, Sam1; Sam2; TM3, Sb* ry^{RK}*/H, FM4/Sam1; Sam2;* ry^{506}, and *CyO,TM6/Xa.* All fly stocks were maintained on molasses-yeast-agar medium at 25° and 70% humidity, on a 12-hr light-dark cycle, with the exception of the transgenic *scrib* stock, which was raised at 18°.

Behavior assays: Three- to 5-day-old adults were divided into single-sex sets of five individuals and deprived of food for 3 hr before testing them for avoidance responses to 0.3% (v/v) benzaldehyde (Sigma Aldrich, St. Louis), as previously described (Anholt *et al.* 1996). The number of individuals in the compartment away from the odorant source was recorded at 5-sec intervals. Ten consecutive observations were averaged to generate the avoidance score for a single replicate. The mean avoidance score for a genotype was calculated by averaging avoidance scores obtained over multiple replicates. Statistically significant differences in avoidance scores between genotypes were assessed using Student's *t*-test.

Larval olfactory behavior was quantified by modifying a previously described assay (Heimbeck *et al.* 1999). To prevent diffusion of odorants through the medium and eliminate larval gustatory responses, filter paper discs saturated with 2 μl of undiluted odorants (either benzaldehyde or isoamylacetate) or distilled water were placed on the lids of 1.5-ml microcentrifuge tubes. The lids were positioned diametrically opposite each other on an 85-mm petri dish containing 10 ml of 1.2% agarose. Ten third instar feeding larvae were placed at the center of the petri dish and the number of individuals within a 10-mm radius from the odorant source was recorded for seven consecutive 30-sec intervals and averaged over 10 replicates for benzaldehyde and 11 replicates for isoamylacetate. Two-way analysis of variance was used to detect differences in the kinetics of odor-guided behavior between genotypes.

Larval motility was quantified by a locomotion assay (Yang *et al.* 2000). Path lengths of individual larvae were traced over a 60-sec time period, in the absence of odorants. Wild type and mutants were compared using Student's *t*-test.

Northern blot analyses: Total RNA (60–100 μg) was isolated from whole flies or larvae using TRIzol reagent (GIBCO BRL, Gaithersburg, MD), size fractionated by 1% formaldehyde gel electrophoresis, and transferred onto nylon membranes (Hybond-XL, Amersham Radiochemicals, Arlington Heights, IL). Blots were hybridized sequentially with [^{32}P]dCTP-labeled full-length (6 kb) *scrib* and β-actin cDNA probes. Hybridizations were performed as described previously (Kulkarni *et al.* 2002). Hybridizing bands were detected in a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Western blot analyses: Homogenates from 6–10 fly equivalents were prepared by homogenization in 50 mm Tris-HCl, pH 6.8, 150 mm NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet NP-40, 0.02% NaN_3, supplemented with a cocktail of protease inhibitors including phenylmethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A, and iodoacetamide. Samples were subjected to polyacrylamide gel electrophoresis in SDS on 4–15% gradient gels and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were blocked with 1% (w/v) nonfat milk in 10 mm sodium phosphate buffer, 100 mm NaCl, 0.05% Tween 20, pH 7.4, and incubated with a 1000-fold dilution of rabbit antibodies, raised against the C-terminal Scrib peptide DMRNPLDEIEAVFRS, or preimmune serum. Immunoreactive bands were visualized using a horseradish peroxidase-conjugated goat-anti-rabbit antibody with a chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ) and exposed to Kodak X-Omat AR film.

cDNA library screening: cDNA libraries were constructed by cloning reverse-transcribed adult head poly(A)$^+$ RNA into the Lambda ZAP Express vector according to the manufacturer's directions (Stratagene, Cedar Creek, TX). A digoxigenin-labeled 6-kb *scrib* cDNA was used to probe the library. Among 2×10^5 recombinants, 11 hybridizing plaques were identified and their inserts were sequenced using T7 and SP6 primers.

***In situ* hybridization:** *In situ* hybridization was performed on 12-μm-thick formalin-fixed and paraffin-embedded sections through adult heads with heat-denatured digoxigenin-labeled riboprobes (Boehringer Mannheim, Indianapolis) corresponding to the sense and antisense strands of a 6-kb *scrib* cDNA, exactly as described previously (Kulkarni *et al.* 2002). Hybridization products were visualized with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals, Indianapolis), using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as substrates. Images were digitally captured and processed with Adobe Photoshop.

Immunohistochemistry: Randomly oriented heads of 3- to 10-day-old male or female flies were fixed overnight in 10% buffered formalin and embedded in paraffin. Following deparaffinization and rehydration, 12-μm-thick sections were blocked with 1% bovine-serum albumin and incubated with a 500-fold dilution of antiserum for 16 hr at 4°. Immunoreactive products were visualized using a horseradish peroxidase-labeled secondary goat-anti-rabbit antibody with H_2O_2 and 3,3-diaminobenzidine as chromogenic substrates.

RESULTS

Phenotypic characterization of *smi97B*: The *smi97B* mutation is one of the strongest *smi* mutations among the set of previously identified *smi* lines (Anholt *et al.* 1996). The mutation is recessive: olfactory ability, quantified by avoidance responses to benzaldehyde, was reduced in *smi97B* homozygotes, compared to the *P*-element-free coisogenic host strain, *Sam;* ry^{506} (*Sam*; $P < 0.0001$), whereas *smi97B/Sam* heterozygotes displayed avoidance responses that were indistinguishable from wild type ($P = 0.56$; Figure 1A). Furthermore, the magnitude of the mutational effect of *smi97B* was sexually dimorphic ($P < 0.05$); even at high odorant concentrations, males were hyposmic, while females were anosmic (Figure 1B).

To determine whether *smi97B* flies experience smell impairment throughout their life cycle, we examined

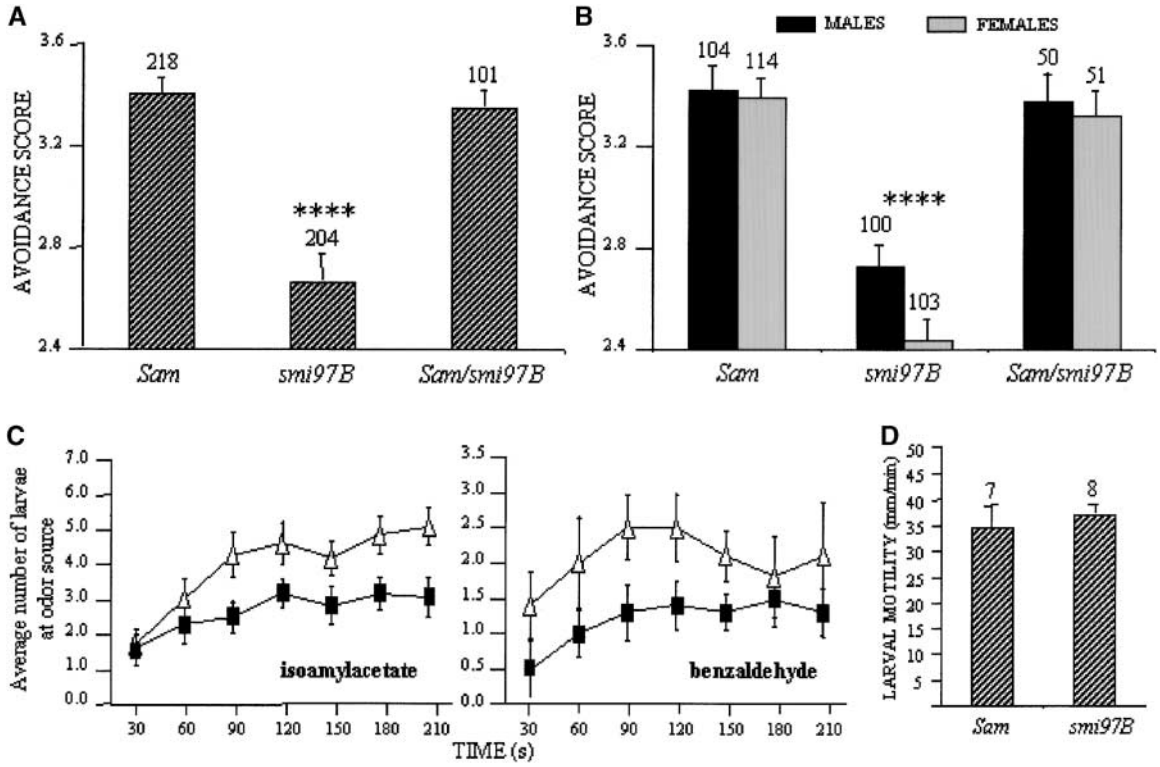

FIGURE 1.—Phenotypic characterization of *smi97B*. (A) Avoidance scores of *Sam*, *smi97B*, and *Sam/smi97B* to benzaldehyde. (B) Avoidance scores of homozygous *smi97B* males and females showing sexual dimorphism ($P < 0.05$). (C) Larval olfactory responses of *smi97B* (■) and *Sam* (△) to isoamylacetate and benzaldehyde. Numbers of larvae at the odorant source were averaged over 10 replicates for benzaldehyde and 11 replicates for isoamylacetate. Error bars indicate SEM. (D) Larval motility in the absence of odorants. Bar graphs in A, B, and D represent mean scores with error bars indicating SEM. Number of replicates for each data set is noted above the error bars. ****$P < 0.0001$.

whether larvae also display aberrant olfactory responsiveness. Since larvae are attracted to most odorants, even those that elicit avoidance behavior in adults, we compared the kinetics of odor-guided responses of wild-type and *smi97B* third instar feeding larvae toward benzaldehyde and isoamylacetate (Figure 1C). The effect of genotype in the two-way analysis of variance was significant for both odorants ($P < 0.0001$), but time and genotype × time interaction terms were not significant for either odorant. Larval motility in the absence of an odor cue was not significantly different between the two genotypes ($P = 0.57$; Figure 1D). Thus, the *smi97B* mutation causes olfactory deficits in both larvae and adults.

***smi97B* is a homozygous viable allele of *scrib*:** We sequenced the genomic fragment flanking the 3′ end of the *P[lArB]* element and determined the insertion site to be 1084 bp upstream of the open reading frame of *scrib* (Figure 2A). *scrib* is a pleiotropic gene essential for localization of polarity determinants in developing epithelia (BILDER and PERRIMON 2000), regulation of cellular growth and proliferation (BILDER *et al.* 2000), organization of fat bodies involved in immune responsiveness (WU *et al.* 2001), and synaptic maturation and modulation of short-term plasticity at the larval neuromuscular junction (ROCHE *et al.* 2002). Here, we show that *smi97B* is an allele of *scrib*.

First, we mapped the *smi97B* mutation to the region of the third chromosome including *scrib*. *Df(3R)Tl-X* (breakpoints 97B2;97D2), which uncovers *scrib*, failed to complement *smi97B*; hemizygotes generated by crossing *Df(3R)Tl-X* to *smi97B* displayed reduced avoidance responses compared to *Df(3R)Tl-X/Sam* controls ($P < 0.0001$; Figure 2B). The smell-impaired phenotype of *Df(3R)Tl-X/smi97B* hemizygotes was also sex specific, with significantly more smell impairment in females than in males ($P < 0.0005$).

Next, to demonstrate that olfactory deficits in *smi97B* arise from the *P[lArB]* element and not a linked mutation, we showed that precise excision of *P[lArB]* restores the wild-type phenotype; avoidance responses of precise excision alleles, like $smi97B^{16A}$, were not significantly different from *Sam* ($P = 0.07$; Figure 3, A and B). Further, mutations generated by imprecise excision of *P[lArB]* provided evidence for sex-specific regulation of *scrib*. The $smi97B^{15A}$ mutation contains a 3.6-kb *P[lArB]* fragment at the original insertion site that resulted in male-specific olfactory deficits (Figure 3, A–C): $smi97B^{15A}$ males were smell impaired compared to controls ($P < 0.005$), while olfactory responses in females were not

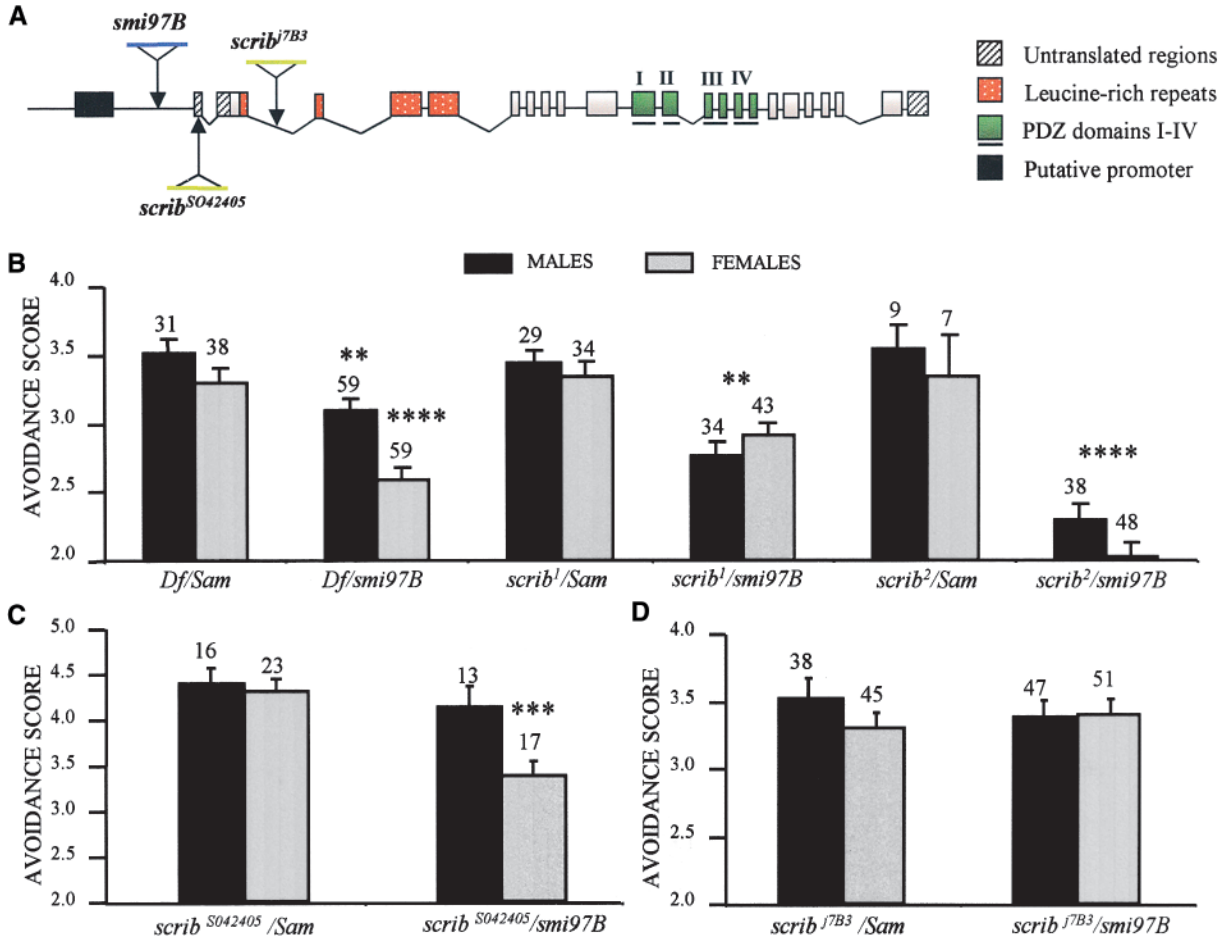

FIGURE 2.—Complementation tests with *scrib* alleles. (A) Schematic representation of the *scrib* gene showing the *P*-element insertion sites in *smi97B*, $scrib^{S0242405}$, and $scrib^{j7B3}$. (B) Avoidance responses of F_1 progeny generated by crossing *Df(3R)Tl-X* and null mutations, $scrib^1$ and $scrib^2$, to *Sam* and *smi97B*. *Df(3R)Tl-X*, $scrib^1$, and $scrib^2$ fail to complement the smell-impaired phenotype of *smi97B*. (C and D) Interallelic complementation between *smi97B* and *P[lacW] scrib* alleles. Avoidance responses of F_1 progeny generated by crossing $scrib^{S042405}$ and $scrib^{j7B3}$ to *Sam* and *smi97B* reveal that the smell-impaired phenotype of *smi97B* was complemented by $scrib^{S042405}$ only in males and by $scrib^{j7B3}$ in both males and females. Avoidance scores of *smi97B* heterozygotes are compared to wild-type, *Sam* heterozygotes for each cross. Bar graphs in B and C represent mean avoidance scores with error bars indicating SEM. Number of replicates for each data set is noted above the error bars. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

statistically different from those in wild type ($P = 0.19$). In contrast, a 2.5-kb *P[lArB]* insertion at the same site in $smi97B^{2A}$ was correlated with mild hyposmia in females ($P < 0.05$), but not in males ($P = 0.86$; Figure 3, A–C). In agreement with the sexually dimorphic phenotype, *scrib* transcripts were markedly reduced in $smi97B^{15A}$ males, but not in females, whereas transcriptional differences could not be resolved in $smi97B^{2A}$, in line with the subtle female-specific phenotype of this imprecise revertant (Figure 3D).

To further implicate *scrib* in olfactory behavior, we conducted complementation tests with previously identified *scrib* alleles. Two null alleles, $scrib^1$ and $scrib^2$, failed to complement the smell-impaired phenotype of *smi97B* (Figure 2B). Avoidance responses of $scrib^1/smi97B$ and $scrib^2/smi97B$ heterozygotes were significantly lower than those of the $scrib^1/Sam$ ($P < 0.01$) and $scrib^2/Sam$ ($P < 0.0001$) controls. We did not, however, observe sexual dimorphism in smell impairment, possibly due to the disparate genetic backgrounds of the *scrib* stocks and *smi97B*. We also tested $scrib^{S0421405}$ and $scrib^{j7B3}$ alleles, which contain *P[lacW]* insertions in the 5′ untranslated region of *scrib* and the second intron, respectively (Figure 2A). Avoidance responses of $scrib^{S0421405}/smi97B$ females were significantly lower than those of $scrib^{S0421405}/Sam$ females ($P < 0.0005$), while male responses were not significantly different from those of control males ($P = 0.38$; Figure 2C). Hence, $scrib^{S0421405}$ failed to complement the olfactory deficit caused by *smi97B*, but only in females. However, $scrib^{j7B3}$ fully complemented the smell-impaired phenotype of *smi97B*; avoidance responses of $scrib^{j7B3}/smi97B$ flies were not significantly different from those of $scrib^{j7B3}/Sam$ controls ($P = 0.96$; Figure 2D). Interallelic complementation is consistent

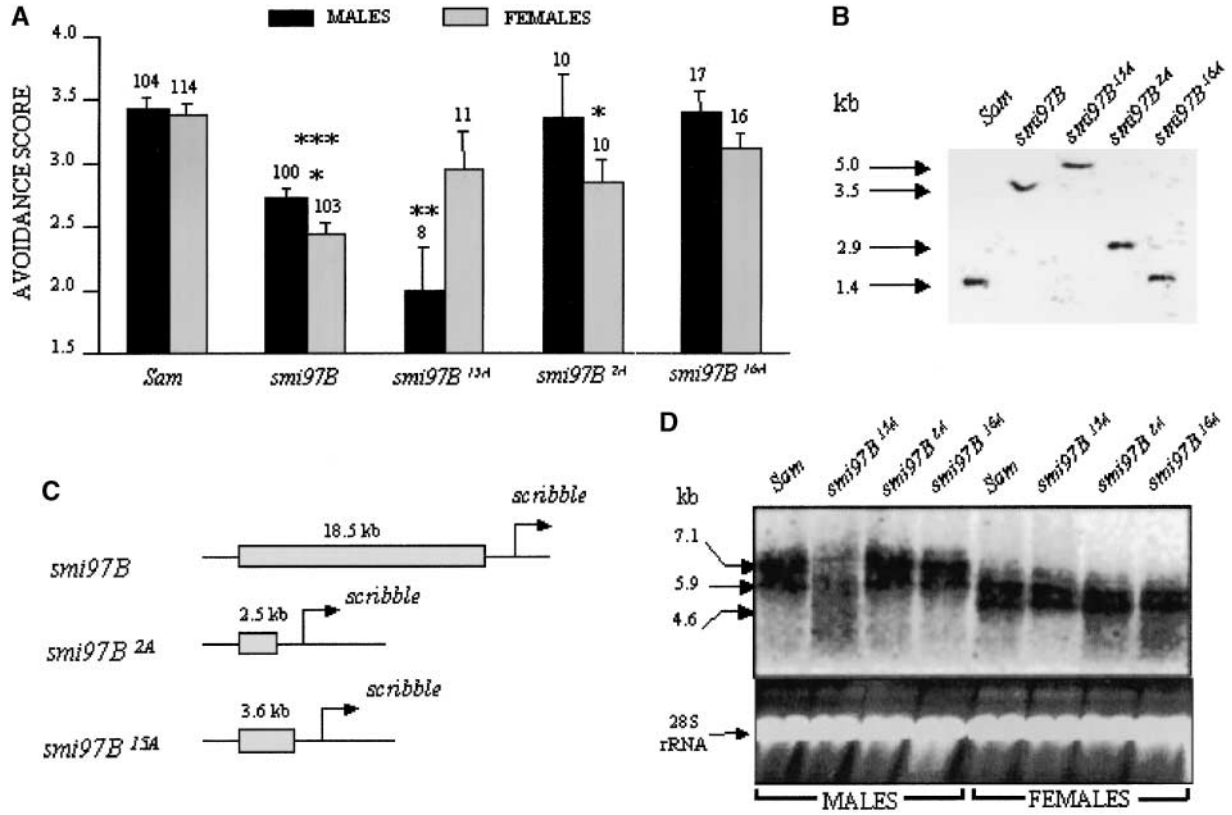

FIGURE 3.—Excision alleles generated by the mobilization of *P[lArB]* from *smi97B*. (A) Avoidance responses of *Sam*, *smi97B*, excision alleles $smi97B^{15A}$ and $smi97B^{2A}$, and phenotypic revertant $smi97B^{16A}$. Bar graphs represent mean avoidance scores with error bars indicating SEM. Number of replicates for each data set is noted above the error bars. $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$. (B) Molecular analysis of excision lines. Southern blot of *Hin*dIII-digested genomic DNA from *Sam*, *smi97B*, $smi97B^{15A}$, $smi97B^{2A}$, and $smi97B^{16A}$, probed with a [^{32}P]dCTP-labeled 0.54-kb genomic fragment adjacent to the 3′ end of the *P[lArB]* element. The 18.5-kb *P[lArB]* element in *smi97B* generates a 3.5-kb *Hin*dIII-digested fragment that hybridizes to the radiolabeled probe, compared to the wild-type 1.4-kb fragment, also observed in the phenotypic revertant, $smi97B^{16A}$. The 5- and 2.9-kb hybridizing fragments in $smi97B^{15A}$ and $smi97B^{2A}$ indicate imprecise excision of the *P[lArB]* element. (C) Schematic representation of *P[lArB]* and its fragments inserted 1084 bp upstream of *scrib*, in *smi97B*, $smi97B^{15A}$, and $smi97B^{2A}$. Arrows show the transcription start site of *scrib*. (D) Northern blot showing *scrib* transcripts in males and females of *Sam* and excision alleles $smi97B^{15A}$, $smi97B^{2A}$, and $smi97B^{16A}$. Note the reduction in transcript levels in $smi97B^{15A}$ males and differences in transcript sizes between males and females (see also Figure 6). The bottom shows ethidium bromide-stained 28S rRNA bands to control for equal loading.

with alternative splicing of *scrib*, which may involve the generation of sex-specific gene products involved in olfaction.

Finally, we demonstrated rescue of the *smi97B* phenotype by functional complementation with a wild-type *scrib* allele. We used a *scrib* transgene under the *Hsp70* promoter on the *X* chromosome (BILDER and PERRIMON 2000), which we introgressed into the *smi97B* background to avoid confounding background genetic effects on behavior (Figure 4). The transgenic stock, $scrib^{hs}$; *smi97B*, was reared at 18° to prevent activation of the *Hsp70* promoter and subsequent expression of *scrib*. Under these conditions no phenotypic rescue is observed in either sex. When raised at 25°, $scrib^{hs}$;*smi97B* was smell impaired compared to *Sam* ($P < 0.01$), but the phenotype was sexually dimorphic ($P < 0.05$). Although $scrib^{hs}$; *smi97B* females were significantly smell impaired ($P < 0.005$), male responses were not statistically different from *Sam* males ($P = 0.53$; Figure 5A). This indicates that leaky expression of *scrib* at a semipermissive temperature for *Hsp70* is sufficient to rescue olfactory deficits in males, but not in females. When heat-shocked at 37°, avoidance responses of $scrib^{hs}$;*smi97B* females were also restored to those of the parental strain ($P = 0.68$; Figure 5A).

Analysis of *scrib* transcripts: The sexually dimorphic olfactory phenotype of *smi97B* (Figure 1B) and evidence for interallelic complementation (Figure 2D) led us to ask whether males and females express alternative splice variants of *scrib*. Three major RNA species were detected on Northern blots probed with a full-length *scrib* cDNA: a universal 5.9-kb transcript present in adults and to a lesser extent in larvae, a 7.1-kb transcript expressed predominantly in males and larvae of both sexes, and a 4.6-kb female-specific transcript (Figure 6; see also Figure 3D). Transcript levels were correlated with sex-specific behavioral phenotypes, as evidenced by reductions in the 7.1-kb transcript in *smi97B* larvae and the

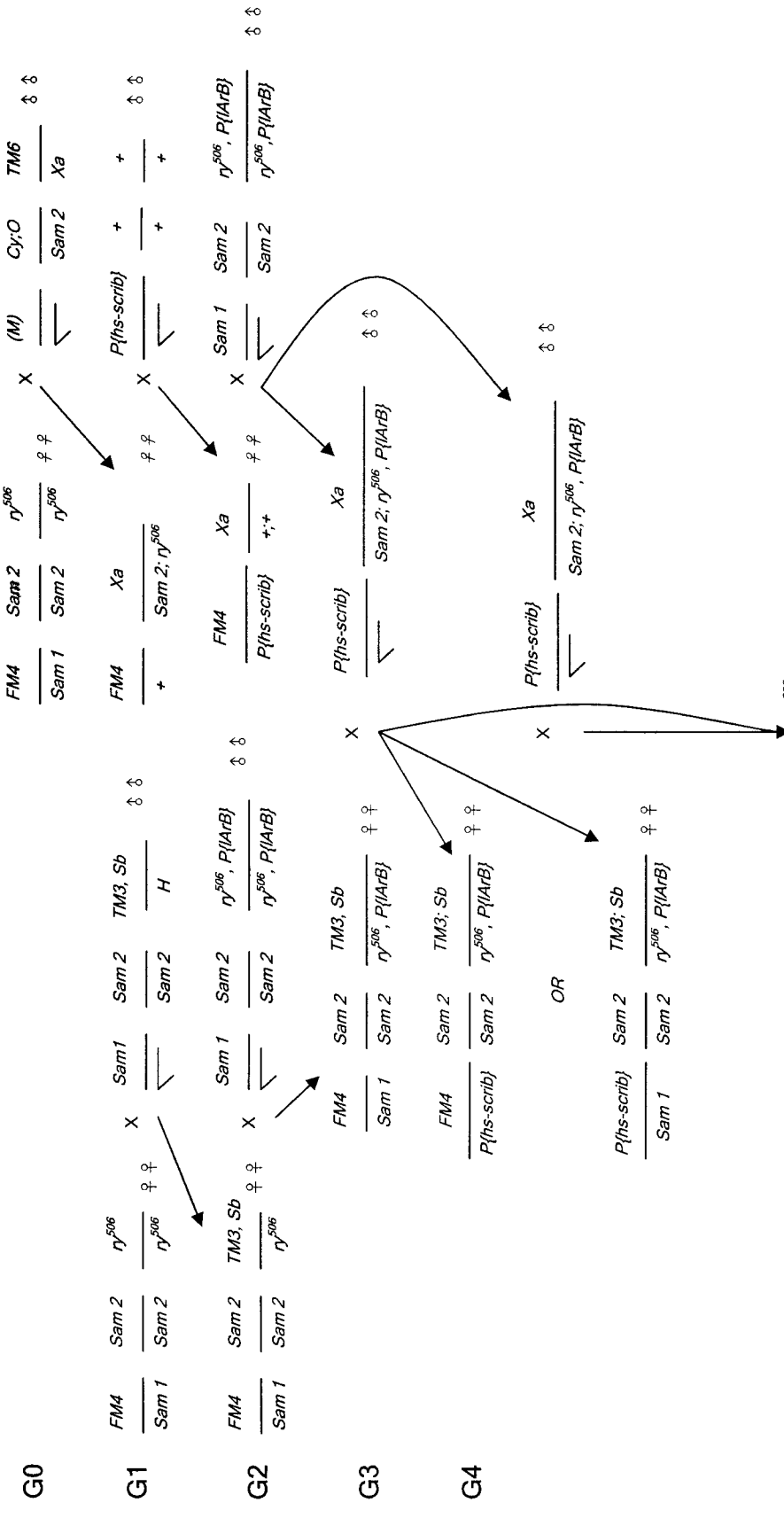

FIGURE 4.—Crossing scheme illustrating the introduction of a full-length *scrib* transgene on the *X* chromosome from a stock generated by BILDER and PERRIMON (2000) into the *smi97B* genetic background.

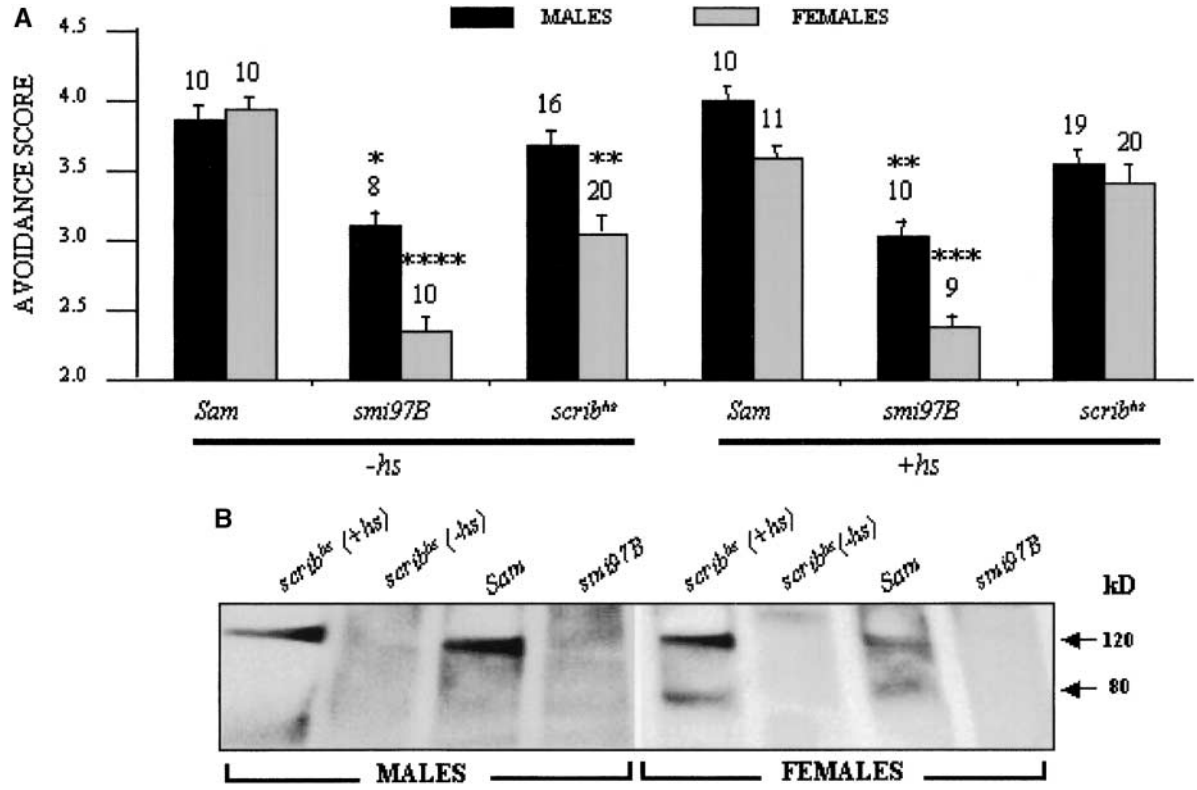

FIGURE 5.—Rescue of *smi97B* using a full-length *scrib* transgene. (A) Behavioral rescue of *smi97B* by a full-length *scrib* transgene. Avoidance scores of *Sam*, *smi97B*, and the *scrib*^{hs} stock (containing an *Hsp-70*-regulated full-length *scrib* cDNA in a homozygous *smi97B* background) recorded at 25° (−*hs*; absence of heat shock) and after heat shock (+*hs*; 37° for 45 min). Partial (male-specific) rescue of the *smi97B* phenotype occurs in the absence of heat shock due to the leaky expression of *scrib* at 25°. The *smi97B* smell-impaired phenotype is rescued after heat shock. Bar graphs represent mean avoidance scores with error bars indicating SEM. Number of replicates for each data set is noted above the error bars. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. (B) Immunochemical analysis of Scrib expression in *Sam*, *smi97B*, and *scrib*^{hs} at 18° and following heat shock.

5.9- and 4.6-kb transcripts in mutant females compared to controls.

To investigate the existence of less prominent variants of *scrib*, we screened a Drosophila head cDNA library with a full-length *scrib* probe. Seven unique clones were identified and sequenced. The sizes of three inserts correspond with splice variants detected on Northern blots (Figure 7, clones *a*, *b*, and *c*). A 7.1-kb clone encoding 16 LRRs, four PDZ domains, and a unique 3′ untranslated exon corresponds to the transcript expressed in males and larvae; a 5.9-kb clone identical to the 7.1-kb fragment, but without the 3′ untranslated region, corresponds to the transcript present in both sexes and larvae; and a 4.6-kb clone encoding 16 LRRs and PDZ domains I and II corresponds to the female-specific transcript. In addition, the 4.6-kb clone also contains a unique coding exon at the 3′ end. The first 285 bases of this exon were shared by four additional clones (Figure 7, clones *d*, *e*, *f*, and *g*), two of which were identical except for the number of LRRs they encode (Figure 7, clones *d* and *e*). In contrast to the 4.6-kb female-specific transcript that encodes only PDZ domains I and II, we identified two clones that encode only PDZ domains III and IV (Figure 7, clones *f* and *g*; note that clone *f* is a partial cDNA sequence). Since LRRs and PDZ domains mediate protein-protein interactions, these differences suggest variability in the composition of protein assemblies recruited by the various Scrib isoforms.

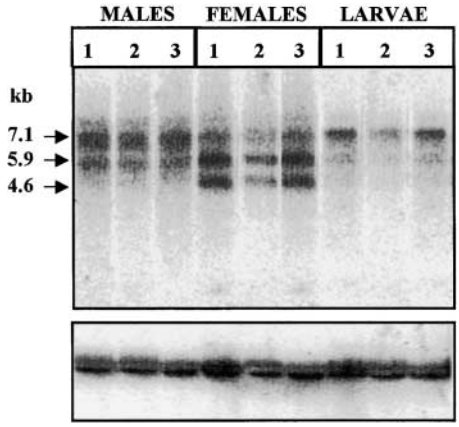

FIGURE 6.—Sex-specific expression of *scrib* in *Sam* (lane 1), *smi97B* (lane 2), and the phenotypic revertant *smi97B*^{16A} (lane 3). A Northern blot of whole-fly extracts probed with a full-length *scrib* cDNA (top) detects three major transcripts (arrows). The same blot was probed with β-actin to control for equal loading of the lanes (bottom). Note the reduction in message in the *smi97B* mutants.

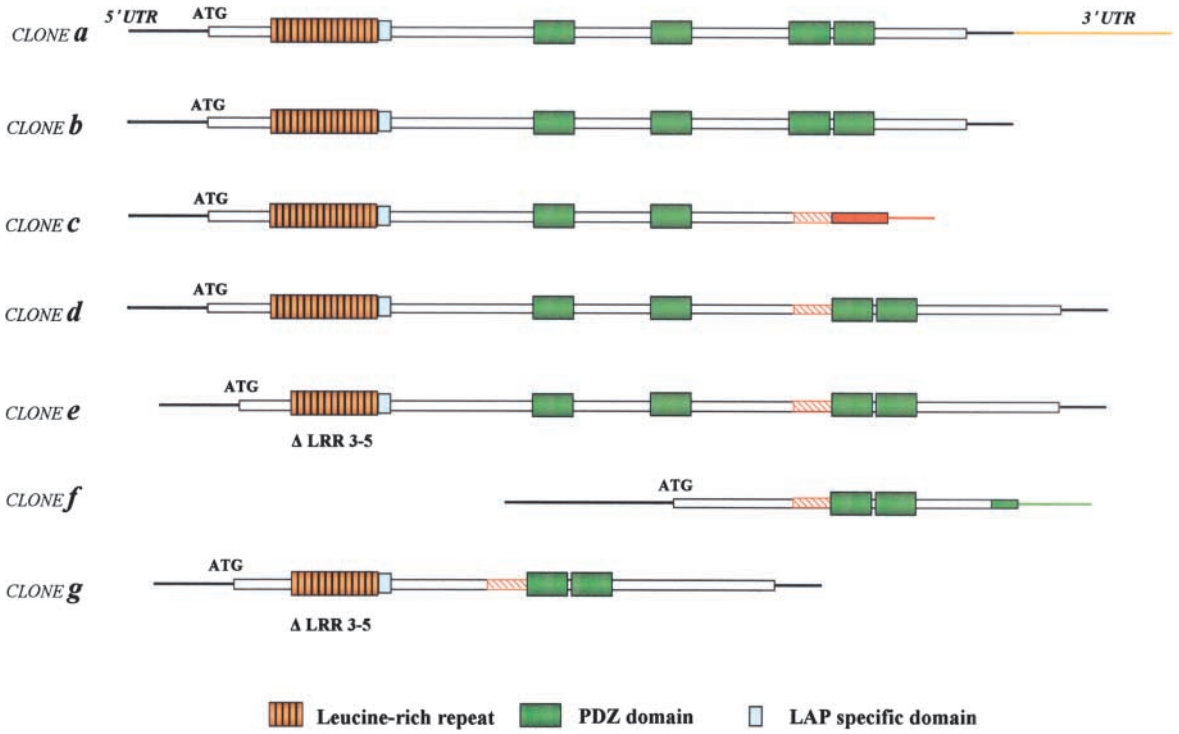

FIGURE 7.—Schematic representation of *scrib* variants encoded by cDNA clones from a head library. Inserts *a–c* correspond to the major mRNA species identified in Figure 6. Isoforms *a* and *c* correspond to male- and female-specific transcripts, respectively. Sequences *d*, *e*, *f*, and *g* were not detected on Northern blots. *UTR* designates untranslated regions. Sequences *e* and *g* carry a deletion in LRRs 3–5. The hatched regions in *c–g*, the red-shaded region and line in *c*, and the blue-shaded region and line in *f* represent alternatively spliced exons, not common to those expressed in sequences *a* and *b*. Sequence *f* represents a truncated transcript.

Monospecific antibodies raised against a carboxyl-terminal peptide of Scrib did not visualize the expected 200-kD polypeptide encoded by the transgene. Instead they detected a 120-kD band in both sexes and an 80-kD female-specific immunoreactive band (Figure 5B), likely due to high sensitivity of the protein in adult flies to proteolysis, which cleaves the expected 200-kD translation product in 120- and 80-kD immunoreactive polypeptides in females, whereas in males the latter fragment is proteolyzed further into smaller fragments (even inclusion of a cocktail of protease inhibitors and performing homogenization in boiling SDS-containing buffer did not preserve the expected 200-kD band). Whereas proteolysis prevents a detailed characterization of sex-specific gene products in adult flies, we could, however, use this antiserum to provide molecular evidence for the heat-shock rescue of smell-impaired *smi97B* by *scrib*^{hs}*;smi97B*. Little or no immunoreactivity was detected in protein extracts from *smi97B* and transgenic flies raised at 18°, whereas the intensity of immunoreactive bands was restored to wild-type levels in extracts from *scrib*^{hs}*;smi97B* following heat shock (Figure 5B). Preincubation of antibodies with the peptides against which they were raised abolished staining on Western blots, verifying specificity (data not shown).

Expression of *scrib* in adult chemosensory organs and central nervous system: Visualization of *scrib* expression in adult tissues with a riboprobe complementary to the *scrib* coding region revealed staining in the third antennal segment and maxillary palps (Figure 8, A–D) and the major olfactory organs of Drosophila as well as in Johnston's organ in the second antennal segment, the primary auditory organ (Figure 8A). Staining was also observed in cortical regions of the brain (Figure 8E). Staining was not observed when hybridizations were performed with sense riboprobes (Figure 8, B and D). No differences in *scrib* expression were observed between males and females under these experimental conditions.

To localize Scrib in CNS projection areas, we performed immunohistochemistry. Staining in the brain was particularly intense in the antennal nerves and the ventrolateral and superior medial protocerebrum (Figure 9A). Widespread deposition of Scrib was also detected in the antennae and maxillary palps (data not shown). Incubation with preimmune serum revealed no staining, demonstrating specificity (Figure 9B).

DISCUSSION

***scrib* is a pleiotropic gene affecting olfactory behavior:** Evidence that *smi97B* is an allele of *scrib* comes from (1) the position of the *P*-element insert between the predicted promoter region and the transcription initiation site (Figure 2A); (2) failure of *Df(3R)Tl-X* and two

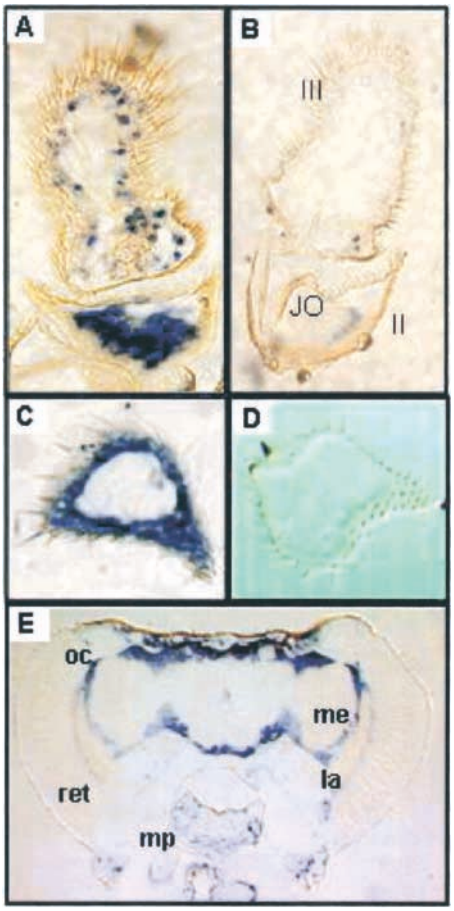

Figure 8.—Localization of *scrib* mRNA in antennae (A and B), maxillary palps (C and D), and the central nervous system (E) of *Sam* flies. *In situ* hybridizations were performed with a digoxigenin-labeled *scrib* anti-sense riboprobe (A, C, and E) and with a sense probe (B and D) to control for nonspecific staining. III, third antennal segment, the main olfactory organ; II, second antennal segment, which houses the mechanosensory neurons of JO, Johnston's organ; me, medulla; la, lamina; oc, optic chiasma; mp, maxillary palp; and ret, retina.

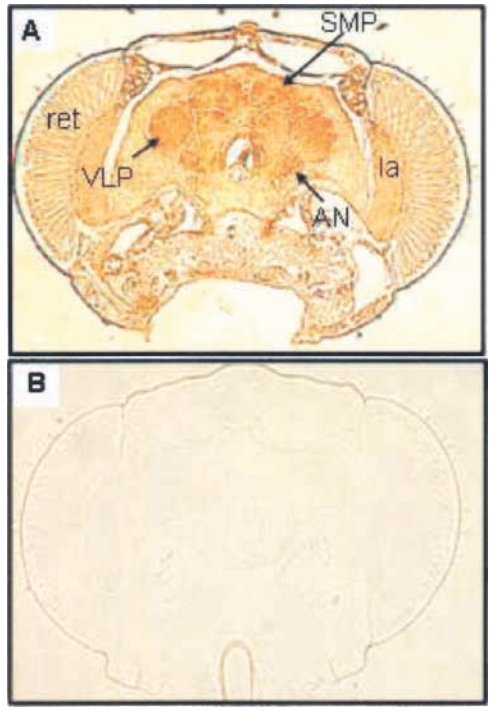

Figure 9.—Immunohistochemical localization of Scrib in frontal sections of the head. (A) Staining with Scrib antiserum. (B) Staining with preimmune serum. VLP, ventrolateral protocerebrum; SMP, superior medial protocerebrum; AN, antennal nerve; ret, retina; and la, lamina.

scrib null alleles to complement the smell-impaired phenotype of *smi97B* (Figure 2B); (3) the restoration of wild-type olfactory phenotype by the precise excision of the *P* element (Figure 3); (4) correlation of *scrib* mRNA and protein expression levels with mutant and wild-type *scrib* phenotypes (Figures 5 and 6); and (5) functional rescue of the olfactory deficit of *smi97B* by a full-length *scrib* construct (Figure 5).

It is of interest to note that differences in expression levels at the mRNA level between the *smi97B* hypomorph and the wild type appear small and are difficult to quantify reliably on Northerns blots (Figure 6). Furthermore, quantitation by quantitative PCR approaches is difficult because of the existence of multiple *scrib* isoforms. At the protein level, however, differences are dramatic and no quantitation is needed as the gene product is distinct in the wild type and virtually absent in *smi97B* (Figure 5B). Subtle reductions in message that translate into large phenotypic effects have been documented previously in studies of behavior (Greenspan 1997; Sokolowski 1998). Minor changes in the activity of a calcium/calmodulin-dependent protein kinase profoundly affect associative learning in Drosophila (Griffith *et al.* 1993). Similarly, in the *foraging* gene as little as a 10% change in the activity of a cyclic GMP-dependent protein kinase causes a dramatic switch in larval feeding behavior (Osborne *et al.* 1997).

Scrib is a LAP (LRR and PDZ) family protein containing 16 LRRs and four Type 1A PDZ domains that bind the consensus sequence S/TXV at carboxyl terminals of proteins (Bilder 2001; Sheng and Sala 2001). These interactions result in the organization and trafficking of multivalent protein complexes (Bilder and Perrimon 2000; Roche *et al.* 2002). In the absence of homozygous viable alleles that enable the characterization of adult phenotypes, descriptions of *scrib* phenotypes have hitherto been restricted to larval and embryonic stages. Interallelic complementation of the sexually dimorphic smell-impaired phenotype of *smi97B* demonstrates functional compartmentalization of alternatively spliced variants of *scrib*. The $scrib^{j7B3}$ insertion in the second intron of *scrib* affects embryonic development, but not olfactory behavior, whereas the $scrib^{S042405}$ insertion disrupts embryonic development as well as female-specific olfactory behavior (Figure 2, A, C, and D).

Sex-specific differences in the penetrance of the smell-impaired phenotype of *smi97B*: Quantitative genetic analyses of olfactory behavior in Drosophila reveal

that genes contributing to naturally occurring phenotypic variation show sex-specific effects (Mackay *et al.* 1996; Fanara *et al.* 2002). Hence, directional selection on olfactory behavior in one sex may not lead to large correlated responses in the other, which facilitates maintenance of genetic variation in a trait under selection pressure. Genotype-by-sex-environment interactions has been documented for a number of complex traits (Long *et al.* 1995; Towne *et al.* 1999; Bowers and Wehner 2001; Corry 2001; Kloting *et al.* 2001); indeed, microarray analyses of the Drosophila transcriptome also revealed that global gene expression levels are most strongly affected by the sex environment (Jin *et al.* 2001). Characterization of imprecise excision alleles $smi97B^{15A}$ and $smi97B^{2A}$ reveals that the size of the *P*-element insertion is critical for sex-specific smell impairment (Figure 3). Sex-specific differences in the penetrance of the smell-impaired phenotype of $smi97B^{15A}$ and $smi97B^{2A}$ are consistent with the observation that $scrib^{hs}$;*smi97B* transgenic flies raised at 25°, a temperature that is semipermissive for the *Hsp70* promoter, show rescue of the olfactory phenotype in males, but not females (Figure 5). Sex-specific splicing of *scrib* in the adult is evident from the expression of a male-biased 7.1-kb transcript and a 4.6-kb female-specific transcript (Figure 6).

Whereas transposon insertions reveal sexually dimorphic smell impairments, chemically induced mutations do not (Figure 2). This may be due either to genetic background differences in the strains, which affect the penetrance of sexually dimorphic effects, or to the fact that point mutations disrupt protein structure, whereas transposon insertions disrupt transcriptional regulation.

Sex-specific gene expression in Drosophila is generally controlled by *doublesex* (*dsx*) and *fruitless* (*fru*), two transcription factors whose activities depend on the splicing regulators *Sex-lethal* (*Sxl*), *transformer* (*tra*), and *transformer 2* (*tra2*; Cline and Meyer 1996; Anand *et al.* 2001; Graveley 2002). However, a recent study, which identified 46 sex-biased genes in fat cells in the head, reported *tra*- and *dsx*-dependent transcripts, but also a *tra*- and *dsx*-independent female-specific transcript, suggesting alternative pathways for the generation of sex-biased gene products (Fujii and Amrein 2002). Whether sex-specific transcriptional regulation of *scribble* is directly or indirectly controlled by genes of the classical sex determination pathway remains to be determined.

The sexually dimorphic effects associated with different *scrib* alleles raises the question whether *in situ* hybridization with different splice variants would resolve male- or female-specific expression patterns. At present, such experiments are hampered by the notion that the isoforms shown in Figure 7 are likely to represent only a subset of all Scribble isoforms and by the likelihood that any given cell may express multiple isoforms. The complexity of the regulation of the *scrib* gene is illustrated by the observation that *lacZ* reporter gene expression in the *smi97B* mutant is restricted to only a few cell groups in the arista at the base of the third antennal segment (Anholt *et al.* 1996), whereas *lacZ* expression in the *p[lac]W* insertion mutant $scrib^{SO42405}$ extends throughout the antenna and no *lacZ* staining at all is detected in the *p[lac]W]* mutant $scrib^{j7B3}$ (data not shown; Figure 2A). Sexually dimorphic expression patterns were not observed in these enhancer trap studies. These observations demonstrate the complex regulation of the *scrib* gene; detailed analysis of transcriptional regulation of *scrib*, therefore, requires further studies.

We thank Nalini Kulkarni, Kellie Robinson, and Akihiko Yamamoto for technical assistance and helpful discussions and David Bilder for generously providing us with fly stocks. This work was supported by grants from the National Institutes of Health (GM-59469, GM-45146, and GM-45344) and the W. M. Keck Foundation.

LITERATURE CITED

Anand, A., A. Villella, L. C. Ryner, T. Carlo, S. F. Goodwin *et al.*, 2001 Molecular genetic dissection of the sex-specific and vital functions of the *Drosophila melanogaster* sex determination gene *fruitless*. Genetics **158:** 1569–1595.

Anholt, R. R. H., R. F. Lyman and T. F. C. Mackay, 1996 Effects of single *P*-element insertions on olfactory behavior in *Drosophila melanogaster*. Genetics **143:** 293–301.

Bellen, H. J., and J. A. Kiger, Jr., 1987 Sexual hyperactivity and reduced longevity of *dunce* females of *Drosophila melanogaster*. Genetics **115:** 153–160.

Benzer, S., 1973 Genetic dissection of behavior. Sci. Am. **229:** 24–37.

Bilder, D., 2001 PDZ proteins and polarity: functions from the fly. Trends Genet. **17:** 511–519.

Bilder, D., and N. Perrimon, 2000 Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature **403:** 676–680.

Bilder, D., M. Li and N. Perrimon, 2000 Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science **289:** 113–116.

Bowers, B. J., and J. M. Wehner, 2001 Ethanol consumption and behavioral impulsivity are increased in protein kinase C gamma null mutant mice. J. Neurosci. **21:** RC180.

Cline, T. W., and B. J. Meyer, 1996 *Vive la différence*: males vs females in flies vs worms. Annu. Rev. Genet. **30:** 637–702.

Corry, D. B., 2001 Sex-related differences in the insulin resistance syndrome. Curr. Hypertens. Rep. **3:** 124–128.

Fanara, J. J., K. O. Robinson, S. M. Rollmann, R. R. H. Anholt and T. F. C. Mackay, 2002 *Vanaso* is a candidate quantitative trait gene for Drosophila olfactory behavior. Genetics **162:** 1321–1328.

Fox, A. N., R. J. Pitts, H. M. Robertson, J. R. Carlson and L. J. Zwiebel, 2001 Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. Proc. Natl. Acad. Sci. USA **98:** 14693–14697.

Fujii, S., and H. Amrein, 2002 Genes expressed in the *Drosophila* head reveal a role for fat cells in sex-specific physiology. EMBO J. **21:** 5353–5363.

Graveley, B. R., 2002 Sex, AGility, and the regulation of alternative splicing. Cell **109:** 409–412.

Greenspan, R. J., 1997 A kinder, gentler genetic analysis of behavior: dissection gives way to modulation. Curr. Opin. Neurobiol. **7:** 805–811.

Griffith, L. C., L. M. Verselis, K. M. Aitken, C. P. Kyriacou, W. Danho *et al.*, 1993 Inhibition of calcium/calmodulin-dependent protein kinase in Drosophila disrupts behavioral plasticity. Neuron **10:** 501–509.

Heimbeck, G., V. Bugnon, N. Gendre, C. Haberlin and R. F.

Stocker, 1999 Smell and taste perception in *Drosophila melanogaster* larva: toxin expression studies in chemosensory neurons. J. Neurosci. **19:** 6599–6609.

Hirsch, J., 1967 *Behavior-Genetic Analysis*, McGraw-Hill, New York.

Jin, W., R. M. Riley, R. D. Wolfinger, K. P. White, G. Passador-Gurgel *et al.*, 2001 The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster*. Nat. Genet. **29:** 389–395.

Kloting, I., P. Kovacs and J. Van Den Brandt, 2001 Sex-specific and sex-independent quantitative trait loci for facets of the metabolic syndrome in WOKW rats. Biochem. Biophys. Res. Commun. **284:** 150–156.

Kulkarni, N. H., A. H. Yamamoto, K. O. Robinson, T. F. C. Mackay and R. R. H. Anholt, 2002 The DSC1 channel, encoded by the *smi60E* locus, contributes to odor-guided behavior in *Drosophila melanogaster*. Genetics **161:** 1507–1516.

Li, M., J. Marhold, A. Gatos, I. Torok and B. M. Mechler, 2001 Differential expression of two scribble isoforms during Drosophila embryogenesis. Mech. Dev. **108:** 185–190.

Long, A. D., S. L. Mullaney, L. A. Reid, J. D. Fry, C. H. Langley *et al.*, 1995 High resolution mapping of genetic factors affecting abdominal bristle number in *Drosophila melanogaster*. Genetics **139:** 1273–1291.

Mackay, T. F. C., 2001 Quantitative trait loci in Drosophila. Nat. Rev. Genet. **2:** 11–20.

Mackay, T. F. C., J. B. Hackett, R. F. Lyman, M. L. Wayne and R. R. H. Anholt, 1996 Quantitative genetic variation of odor-guided behavior in a natural population of *Drosophila melanogaster*. Genetics **144:** 727–735.

Mathew, D., L. S. Gramates, M. Packard, U. Thomas, D. Bilder *et al.*, 2002 Recruitment of scribble to the synaptic scaffolding complex requires GUK-holder, a novel DLG binding protein. Curr. Biol. **12:** 531–539.

Mombaerts, P., 2001 The human repertoire of odorant receptor genes and pseudogenes. Annu. Rev. Genomics Hum. Genet. **2:** 493–510.

Nadeau, J. H., and W. N. Frankel, 2000 The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. Nat. Genet. **25:** 381–384.

Osborne, K. A., A. Robichon, E. Burgess, S. Butland, R. A. Shaw *et al.*, 1997 Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. Science **277:** 834–836.

Price, J. L., J. Blau, A. Rothenfluh, M. Abodeely, B. Kloss *et al.*, 1998 *double-time* is a novel Drosophila clock gene that regulates PERIOD protein accumulation. Cell **94:** 83–95.

Roche, J. P., M. C. Packard, S. Moeckel-Cole, and V. Budnik, 2002 Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble. J. Neurosci. **22:** 6471–6479.

Shaver, S. A., C. J. Varnam, A. J. Hilliker and M. B. Sokolowski, 1998 The *foraging* gene affects adult but not larval olfactory-related behavior in *Drosophila melanogaster*. Behav. Brain Res. **95:** 23–29.

Sheng, M., and C. Sala, 2001 PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. **24:** 1–29.

Sokolowski, M. B., 1998 Genes for normal behavioral variation: recent clues from flies and worms. Neuron **21:** 463–466.

Toma, D. P., K. P. White, J. Hirsch and R. J. Greenspan, 2002 Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait. Nat. Genet. **31:** 349–353.

Towne, B., L. Almasy, R. M. Siervogel and J. Blangero, 1999 Effects of genotype × sex interaction on linkage analysis of visual event-related evoked potentials. Genet. Epidemiol. **17** (Suppl. 1): S355–S360.

Tully, T., 1996 Discovery of genes involved with learning and memory: an experimental synthesis of Hirschian and Benzerian perspectives. Proc. Natl. Acad. Sci. USA **93:** 13460–13467.

Vosshall, L. B., 2001 The molecular logic of olfaction in Drosophila. Chem. Senses **26:** 207–213.

Warr, C., P. Clyne, M. De Bruyne, J. Kim and J. R. Carlson, 2001 Olfaction in Drosophila: coding, genetics and e-genetics. Chem. Senses **26:** 201–206.

Wu, L. P., K. M. Choe, Y. Lu and K. V. Anderson, 2001 Drosophila immunity: genes on the third chromosome required for the response to bacterial infection. Genetics **159:** 189–199.

Yang, P., S. A. Shaver, A. J. Hilliker and M. B. Sokolowski, 2000 Abnormal turning behavior in Drosophila larvae: identification and molecular analysis of *scribbler* (*sbb*). Genetics **155:** 1161–1174.

Zhang, X., and S. Firestein, 2002 The olfactory receptor gene superfamily of the mouse. Nat. Neurosci. **5:** 124–133.

Communicating editor: K. V. Anderson