The *Drosophila takeout* gene is regulated by the somatic sex-determination pathway and affects male courtship behavior

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The *Drosophila* somatic sex-determination regulatory pathway has been well studied, but little is known about the target genes that it ultimately controls. In a differential screen for sex-specific transcripts expressed in fly heads, we identified a highly male-enriched transcript encoding Takeout, a protein related to a superfamily of factors that bind small lipophilic molecules. We show that sex-specific *takeout* transcripts derive from fat body tissue closely associated with the adult brain and are dependent on the sex determination genes *doublesex* (*dsx*) and *fruitless* (*fru*). The male-specific Doublesex and Fruitless proteins together activate Takeout expression, whereas the female-specific Doublesex protein represses takeout independently of Fru. When cells that normally express *takeout* are feminized by expression of the Transformer-F protein, male courtship behavior is dramatically reduced, suggesting that male identity in these cells is necessary for behavior. A loss-of-function mutation in the *takeout* gene reduces male courtship and synergizes with *fruitless* mutations, suggesting that takeout plays a redundant role with other *fru*-dependent factors involved in male mating behavior. Comparison of Takeout sequences to the *Drosophila* genome reveals a family of 20 related secreted factors. Expression analysis of a subset of these genes suggests that the *takeout* gene family encodes multiple factors with sex-specific functions.

[*Keywords*: Sex determination; courtship behavior; fat body; gene family; *doublesex*; *fruitless*]

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Sexual differentiation affects the form and function of a wide variety of tissues in adult organisms. In *Drosophila*, somatic sexual identity is controlled by a well-studied pathway of regulatory genes in which globally acting factors (i.e., *sis-a*, *sis-b*, *Sxl*, *tra*, *tra-2*) determine the alternative sex-specific products that are synthesized from the *fru* and *dsx* genes. Sex-specific Dsx and Fru proteins then enact sexual differentiation in distinct subsets of somatic tissues (Nagoshi et al. 1988; Ito et al. 1996; Ryner et al. 1996). The male-specific Fru protein is necessary for sexual differentiation within the CNS of the adult fly and appears to be a major factor controlling the sexual differentiation of behavior (Baker et al. 2001). *XY fru* mutants develop with the appearance of normal males, but do not perform normal male courtship behavior and fail to distinguish correctly between males and females (Hall 1994; Taylor et al. 1994; Ryner et al. 1996; Villella et al. 1997; Anand et al. 2001). Somatic sexual differentiation outside of the CNS is controlled primarily by the *dsx* gene. Two alternative sex-specific proteins encoded by this gene (Dsx-M and Dsx-F) specify male and female differentiation, respectively (Burtis and Baker 1989). In the absence of *dsx* function, sexual differentiation in both XX and XY flies is ambiguous, resulting in nearly identical intersexual adults that have both male and female characteristics (Baker and Ridge 1980). Dsx also appears to play a role in the differentiation of some tissues needed for sex-specific behavior, as *dsx* mutant flies have quantitatively reduced ability to perform various aspects of male courtship and lack the ability to produce the sine-song, humming sounds that are part of the courtship song (McRobert and Tompkins 1985; Taylor et al. 1994; Villella and Hall 1996).

Although the regulatory interactions within the sex-determination pathway are well understood, the interactions of these factors with the downstream target genes that the pathway ultimately controls are largely unexplored. The Fru and Dsx proteins both encode transcription factors that function at parallel terminal positions in the known regulatory hierarchy and, thus, are likely to directly control at least some targets. However, as yet, no genes regulated by Fru have been identified. Dsx has been shown to regulate the function of factors that con-

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trol patterning in the genital disc (Keisman and Baker 2001; Keisman et al. 2001; Sanchez et al. 2001), the pigmentation of abdominal cells (Kopp et al. 2000), and the expression of a variety of factors associated with reproductive systems of adults (Chapman and Wolfner 1988), but its only clear direct targets are the female-specific yolk protein genes that are activated by Dsx-F and repressed by Dsx-M (Coschigano and Wensink 1993; Bownes 1994). On the basis of this example, it seems likely that the *dsx* gene can act as a bimodal molecular switch, oppositely affecting target gene expression in males and females. Identification of more targets of the sex-determination pathway would improve our understanding of the function of Dsx, Fru, and other downstream sex-determination regulators. Moreover, analysis of target gene function is likely to lead to insights into molecular processes that determine sex-specific traits. Here, we present evidence that the *takeout* gene is a tissue-specific target of regulation by both Fru and Dsx. Takeout is a member of a large family of secreted factors that bind small lipophiles and was identified previously in several molecular screens as a robust circadian-regulated gene (Sarov-Blat et al. 2000; Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Lin et al. 2002). Interestingly, Takeout is induced in adults by starvation and improves their tolerance to nutrient deprivation. (Sarov-Blat et al. 2000). Through analysis of *takeout* mutants and sexual mosaics, we find that it is also required for normal levels of male courtship behavior. These findings lead us to propose that *takeout* plays a role in integrating information about the organism's sex, nutritional status, and circadian cycle to affect adult male behavior.

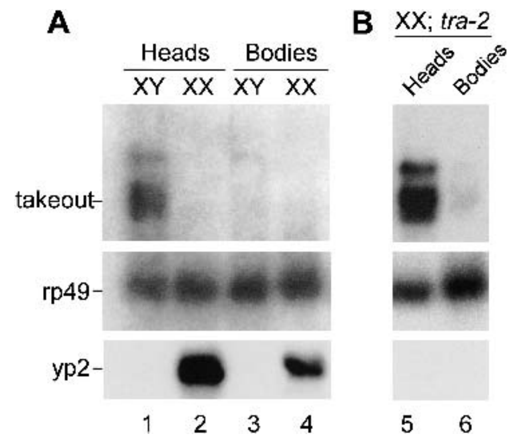

Figure 1. The *takeout* gene is expressed specifically in male heads and is derepressed in *tra-2* mutant chromosomal females. (*Top*) Takeout expression in head and body RNA from nonstarved Canton-S male (XY) and female (XX) flies (*A*) and in *tra-2*^{PM6}/*ra-2*^{PM7} mutant females (*B*) was examined by Northern analysis. The major band corresponding to *takeout* mRNA is indicated. The higher molecular weight band corresponds in size to *takeout* pre-mRNA. (*Middle*) Ribosomal protein 49 (rp49) hybridization to the same blot as a control for amount of RNA loaded. (*Bottom*) Hybridization with a probe for transcripts from the yolk protein 2 gene, which is expressed in female fat body.

Results

Takeout expression is sex-specific in brain-associated fat body and is regulated by Transformer-2

To identify genes under the control of the sex-determination regulatory pathway, we carried out a PCR-based subtractive hybridization screen for sex-specific RNAs expressed in adult fly heads. Head RNA of *tra-2/tra-2*$^{+}$ phenotypically wild-type XX adult females was subtracted against the head RNA of sibling XX *tra-2/tra-2* mutants, and vice versa. The latter flies are transformed into males both somatically and behaviorally (Watanabe 1975; Belote and Baker 1987). One cDNA clone that hybridized preferentially with sequences from phenotypic males was isolated and studied in more detail. Northern blot hybridizations confirmed that this sequence represents a highly male-specific 1.1-kb mRNA that was expressed primarily in adult heads (Fig. 1A). Expression of this mRNA was repressed by Tra-2 in females, as XX *tra-2* mutants expressed levels similar to wild-type males (Fig. 1B). The sequence of the clone was later found to be identical to that of *takeout*, an independently identified gene responsive to circadian rhythms and starvation (Sarov-Blat et al. 2000). The *takeout* gene encodes a secreted protein related to circulating carrier proteins of lipophilic factors, such as the juvenile hormone-binding proteins of other insects. In our initial screen and in the experiments described below, we have used flies grown in parallel, nonstarved cultures that were not entrained to a light-dark cycle. Analysis of RNA prepared from these cultures at different times during the day failed to reveal any significant variation in *takeout* levels, presumably due to their asynchrony (data not shown).

Takeout expression has been reported in the adult brain as well as in the cardia and other segments of the digestive system, in which it is induced by starvation (Sarov-Blat et al. 2000). To identify tissues giving rise to male-specific *takeout* transcripts, we performed RNA in situ hybridizations on parallel serial sections of adult male and female heads. Surprisingly, we did not detect *takeout* RNA within the adult brain using either of two probes from different regions of the *takeout* transcription unit, even when samples were overstained (see Materials and Methods). Instead, both probes detected high levels of RNA in the fat body that surrounds the brain as well as in a dispersed population of cells in the third antennal segment (Fig. 2A,B). Expression was not detected in males homozygous for the *to*^{1} mutation (Fig. 2A, bottom). Comparison of simultaneous hybridizations performed on sections from males and females revealed that fat-body expression was male specific, but that expression in the antennae was not. To confirm this, we dissected antennae from male and female heads and carried out low-cycle RT–PCR (Fig. 2C). This showed that although overall accumulation of *takeout*

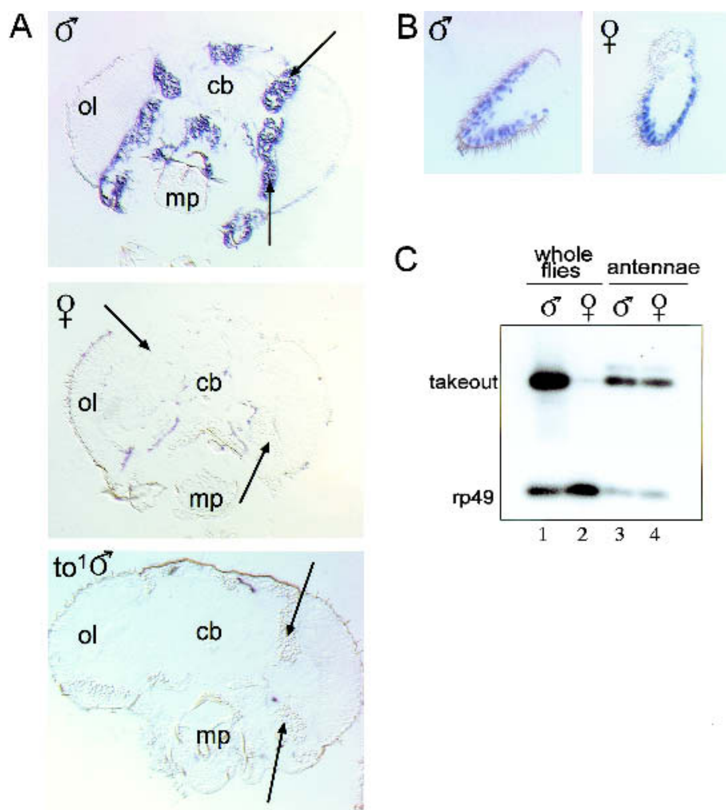

Figure 2. *takeout* RNA is expressed male specifically in brain-associated fat body, whereas expression in the antennae is not sex specific. (*A*,*B*) Frontal sections through male and female Canton-S heads were hybridized in situ with a *takeout* riboprobe originating from the 3′ untranslated region of the gene. Expression in the fat body (arrows) was observed in males, but not in females. Hybridization to the *takeout* probe was absent in *takeout* (*to*[1]) males. (*B*) Expression in the antennae was observed in both sexes. For reference, the positions of the central brain (cb), optical layers of the brain (ol), and mouthparts (mp) are indicated. (*C*) *takeout* RT–PCR on RNA from isolated antennae as well as whole male and female Canton-S flies is shown. Low-cycle PCR was performed with *takeout* and *rp49* primers. The products were detected by Southern blotting and hybridization with an internal oligonucleotide probe.

RNA in whole flies is highly male biased, no difference was apparent in male and female antennal RNA levels. We conclude that antenna-derived *takeout* messages account for only a small fraction of all *takeout* RNA, and that sex-specific expression of *takeout* in adult heads derives primarily from the fat body. Thus, the sex-specific regulation of *takeout* varies by tissue type.

Other members of the takeout *gene family are expressed sex specifically*

Takeout has been shown to have similarity to six other *Drosophila* proteins that are also under circadian control (So et al. 2000; Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Lin et al. 2002). When used to carry out BLAST-P searches of both the translated *Drosophila* genome sequence and the entire set of predicted *Drosophila* proteins in the Berkeley *Drosophila* Genome Project Database Takeout, Adams et al. (2000) identifies a family of 20 related proteins. An alignment of these proteins is shown in Figure 3. The sequences share interspersed regions of conservation that correspond to regions in Takeout noted previously (Sarov-Blat et al. 2000) to have similarity with circulating juvenile hormone-binding proteins (JHBPs) of other insects (Robertson et al. 1999; Vermunt et al. 2001). Although the *takeout* genes are dispersed to several locations in the genome, most are found in clusters of two or three closely linked genes (Fig. 4A). To determine whether other members of the *takeout* family are also expressed in a sex-specific manner, we surveyed the expression of five randomly selected family members (*CG1124*, *CG2016*, *CG5867*, *CG7096*, and *CG11852*) in adult males and females. Although transcripts from three of these genes accumulated equally in both sexes (data not shown), RNAs from *CG5867* and *CG7096* were found to be male enriched in adult heads (Fig. 4B,C). These results support the idea that members of the *takeout* gene family perform sex-specific functions.

Feminization of Takeout-expressing cells disrupts male courtship behavior

Because *takeout* identifies sexually differentiated cells within the adult head, we next asked whether male sexual identity within these cells is important for male-specific courtship behavior. Specific cell types can be sexually transformed from male to female by forcing them to express the female-specific transformer protein (TraF; Ferveur et al. 1995) by use of a tissue-specific promoter. Taking advantage of the yeast GAL4/UAS system, a 1.27-kb *takeout* promoter fragment was placed upstream of the *Gal4* gene and used to drive expression of a *UAS–TraF* transgene. When expression of five independent *takeout–GAL4* insertions was tested by crossing to a strain carrying a *UAS–lacZ* transgene, activity of the promoter was found consistently to be most prominently distributed in a pattern similar to that found in

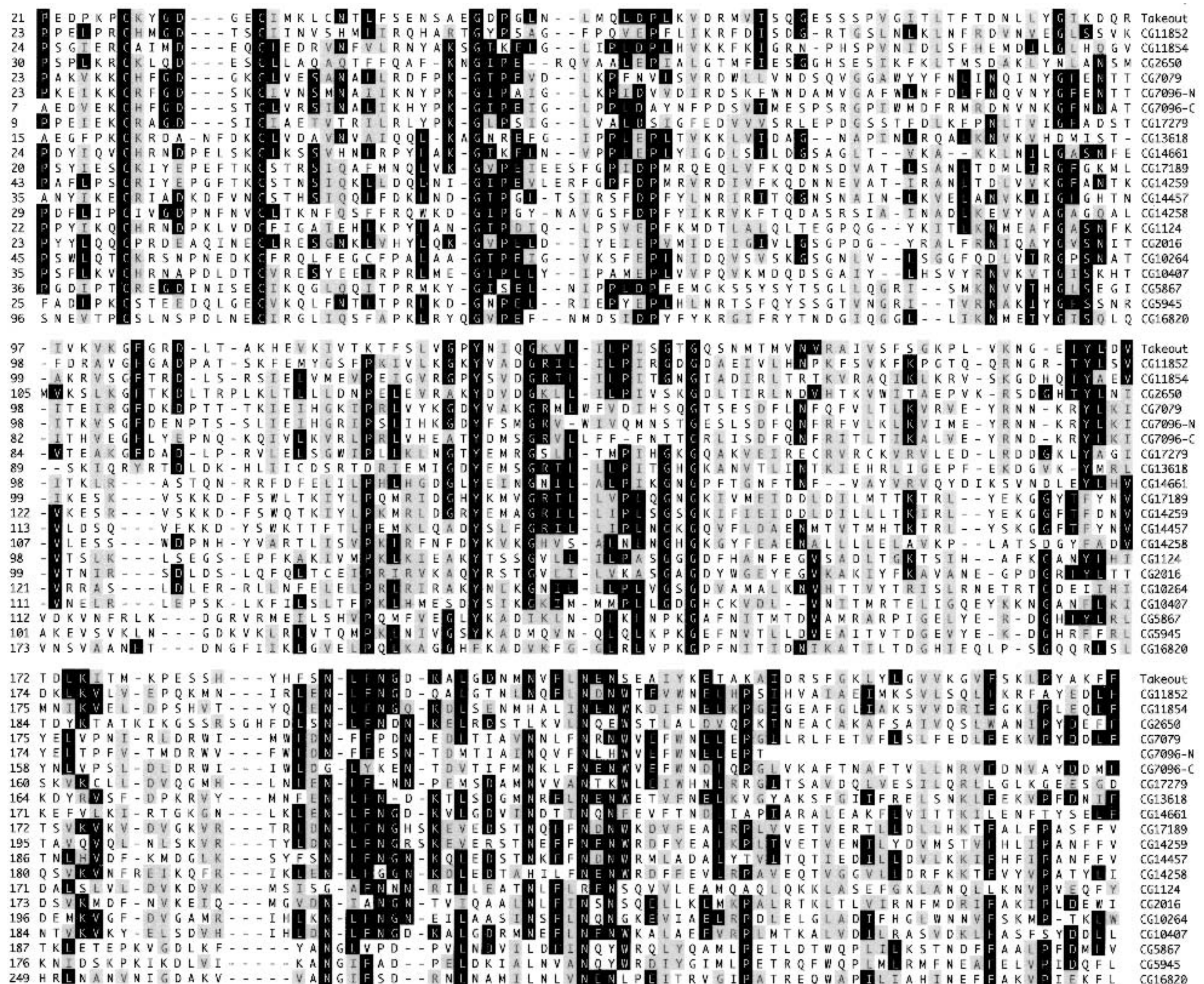

Figure 3. The Takeout family of proteins. An alignment is shown of the conserved protein-coding regions from the 20 different members of the *takeout* gene family encoded by *Drosophila*. Black shading indicates residues that are identical in at least eight members of the family, and gray shading indicates areas of similarity. CG7096-N and CG7096-C denote the N- and C-terminal sequences of the single ORF encoded by CG7096, in which the entire Takeout homology region is duplicated. In most cases, protein sequences are derived from translation of EST clones. In all other cases, sequences are based on the predicted genomic protein-coding regions as annotated by the *Drosophila* genome project (Adams et al. 2000). The sequences shown for CG14661, CG14457, and CG17279 are modified after splice junction reassignment on the basis of sequence alignment with other family members.

our in situ hybridization experiments. Within adult heads, activity was detected in fat body as well as in a subset of cells within the maxillary palps and antennae (Fig. 5A–D). In sections from whole adult fly bodies, a lower level of expression was also detected within the cardia and in fat cells dispersed throughout the abdomen and thorax (data not shown). Although expression of endogenous *takeout* RNA was not observed in maxillary palps, its detection in this experiment is likely due to higher sensitivity of the reporter method relative to RNA in situ hybridization. Notably, promoter activity was never observed in any part of the adult CNS, even when sections were purposely overstained. Whereas the spatial pattern of staining was consistent among the five strains we examined, the sex-specific expression was not. In only one of the lines examined was the expression observed in fat bodies clearly male specific. In other lines, significant levels of expression were observed in both males and females. Because each line represents an independent transgene insertion, we infer that the 1.27-kb *takeout* promoter fragment used is not sufficient to direct sex-specific expression at most insertion sites. This observation was fortunate as the non-sex-specific activity of these *takeout–GAL4* insertions allowed us to circumvent artificial negative feedback regulation in our feminization strategy (see Fig. 5F) that would otherwise result from expression of TraF using a promoter under the negative control of the TraF protein.

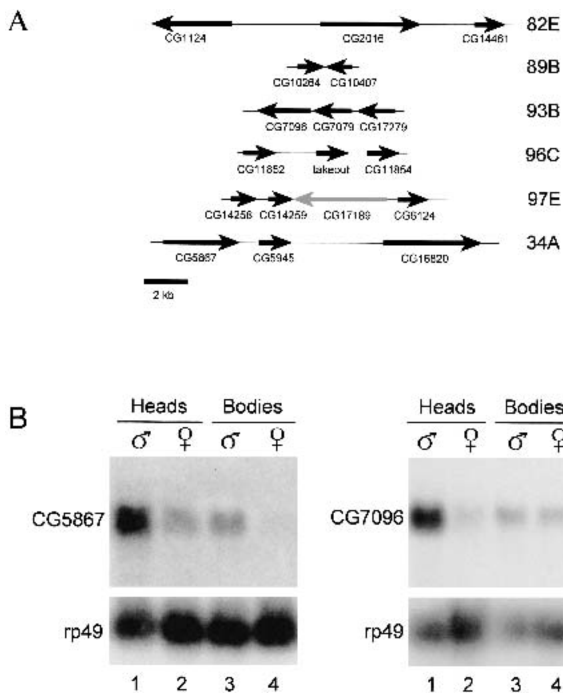

Figure 4. Multiple members of the *takeout* family are expressed sex specifically. (*A*) Organization of *takeout* family gene clusters are diagrammed. Arrows denote the length of each coding region and transcriptional orientation. Exons and introns are not indicated. Names of genes are below the arrows. The gray arrow denotes a gene (*CG17189*) that interrupts a cluster but is not in the *takeout* family. The cytological position of each cluster in the *Drosophila* genome is given at *right*. Three genes at dispersed locations (*CG2645*, *CG14457*, and *CG13618*) are not shown. (*B*) RNA blot hybridization analysis of CG5867 and CG7096 RNA expression in heads and bodies from males and females is shown. CG5867 is male enriched in both tissues, whereas CG7096 is male enriched only in heads. Hybridization of the same blot with rp49 mRNA is shown at *bottom*.

Adult males expressing TraF under the direction of *takeout–GAL4* transgenes were observed for their ability to court wild-type females in a mating chamber (Hall 1994; Greenspan 1995). Results are shown in Figure 5E, represented as the courtship index (CI), which is a measure of the time a male spends performing any of the steps of courtship during a fixed observation period. The *takeout–GAL4*/*UAS–traF* flies from all three driver lines tested gave markedly reduced courtship indices in relation to controls, reflecting the fact that these males spent much less time courting females. Feminization directed by the *takeout* promoter severely lowered the probability that a male would court or sustain courtship beyond the initial steps of orienting and following. Although, on occasion, all steps of courtship can be carried out by such males. These results indicate that the *takeout* gene is active in sexually differentiated cell types that play an important role in promoting male courtship behavior.

The requirement for male identity of *takeout*-expressing cells is further supported by comparison of the results obtained in the different driver lines tested. As an indicator of feminization, we looked at levels of endogenous *takeout* RNA (Fig. 5G). If *takeout*-expressing cell types are completely feminized, we expect that endogenous RNA expression will be greatly reduced. Feminization driven by nonsex-specific *takeout–GAL4* drivers (Fig. 5G, lines 1,2) virtually eliminated *takeout* RNA expression. This argues that these lines are feminized in cell types that normally express *takeout*. In contrast, feminization by the male-specific *takeout–GAL4* driver (Fig. 5G, line 3) was incomplete, and endogenous *takeout* RNA was only slightly reduced in amount. This is presumably due to the anticipated negative feedback regulation that reduces the level of TraF expressed in these flies (Fig. 5F). Feminization, as measured in this way, was well correlated with the ability of these flies to court females. Flies from lines 1 and 2 were more affected than those from line 3 (Fig. 5E, cf. red bars). We conclude that the degree of feminization of *takeout*-expressing cell types is related to the ability of males to perform courtship behavior.

A mutation in takeout *interacts genetically with* fruitless *in male courtship*

To determine whether *takeout* function is required for male mating behavior, we performed courtship assays on *takeout* mutant males. In a previous study (Sarov-Blat et al. 2000), it was shown that a rearranged mutant allele of *takeout* (to^1) is fortuitously carried in a laboratory strain on the ry^{506} third chromosome. We obtained a ry^{506} mutant strain carrying an identical rearrangement, and found that it also fails to express *takeout* RNA (data not shown). Therefore, we refer to this allele as to^1. PCR and Southern blotting experiments on to^1 showed that the deletion found previously (Sarov-Blat et al. 2000) is associated with a chromosome rearrangement breakpoint and located entirely within a region between 39 nucleotides upstream and 494 nucleotides downstream of the *takeout*-translation stop codon (data not shown).

When tested for courtship behavior, ry^{506} to^1 mutant males did not show a reduction in courtship relative to heterozygous siblings (Fig. 6A, cf. lanes 1 and 2). However, *takeout* mutant flies that were also heterozygous for *fru* (ry^{506} fru^4 to^1/ry^{506} fru^+ to^1 and ry^{506} fru^3 to^1/ry^{506} fru^+ to^1) showed a significant reduction in courtship relative to a variety of control genotypes tested (Fig. 6A, lane 3, B, lanes 2,3). No effects of ry^{506} alone on courtship were observed (Fig. 6A). To determine whether the observed effect of *takeout* might be explained by any general sluggishness of the affected genotypes, we carried out short-term activity assays to measure the movement of males in the same chambers used for courtship assays, but without a female present (see Materials and Methods for details). In these assays, the activities of the courtship-defective genotypes *ry* to^1/*ry* fru^3 to^1 (89 ± 4, n = 10) and *ry* to^1/*ry* fru^4 to^1 (90 ± 4, n = 10) were found to be equal to or slightly greater than that of either *ry* to^1

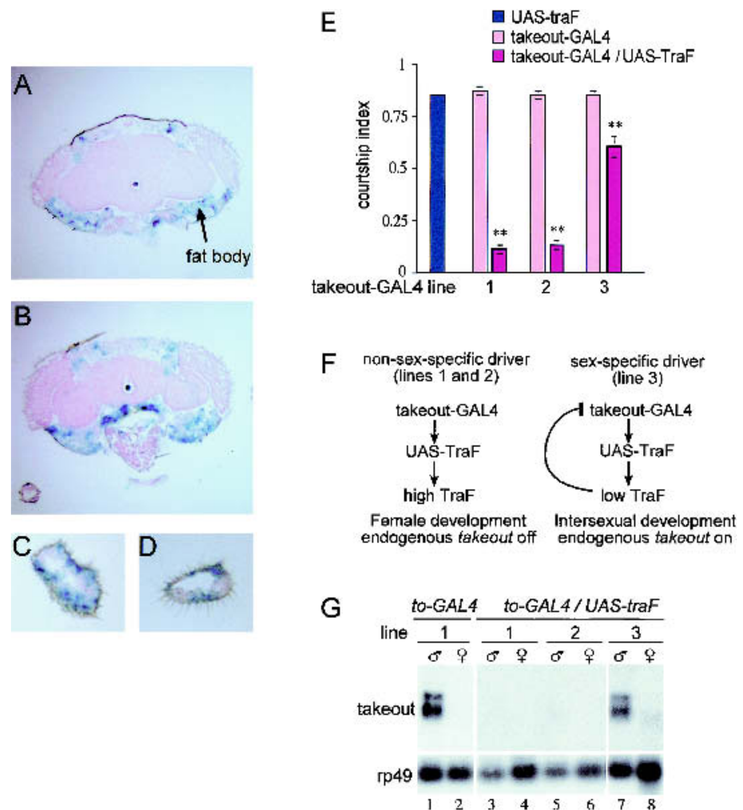

Figure 5. Feminization of Takeout-expressing cells disrupts male courtship behavior. GAL4 activity driven by the *takeout* promoter in sections from adult heads (*A*,*B*), antennae (*C*), and maxillary palps (*D*), was detected using a *UAS–lacZ* reporter gene. Frontal sections of *takeout–GAL4*/*UAS–lacZ* flies were stained with X-gal to detect β-galactosidase activity. Courtship indices of various males toward Canton-S virgin females are shown in *E*. Males carrying the *UAS–traF* (blue bar) or *takeout–GAL4* (pink bars) transgenes individually have significantly higher courtship indices than the *takeout–GAL4*/*UAS–traF* males carrying both transgenes (red bars). The results from three different transgenic *takeout–GAL4* lines are shown (lines *1*,*2*,*3*). $n = 10$ for each group. ** indicates indices that were significantly different from those of parental strains ($p < 0.001$). (*F*) Diagram of the expected negative feedback loop set up in progeny from a cross of *UAS–traF* flies with the male-specific *takeout–GAL4* line. (*G*) Northern analysis of endogenous *takeout* expression in *takeout–Gal4*/*UAS–traF* males and females as a measure for the feminization of *takeout*-expressing cells shows that *takeout* expression is drastically reduced in lines *1* and *2*, but to a lesser degree in line *3*. Expression of endogenous *takeout* RNA in the parental *takeout–Gal4* adults from line *1* is shown for comparison (lanes *1*,*2*).

double homozygous (84 ± 5, n = 10) or heterozygous (76 ± 5, n = 10) control flies that were tested in parallel.

Although courtship of the above mutant flies is quantitatively reduced, it is not absent. The mutant males are capable of all steps of courtship, but perform them less frequently, and seem to lack motivation to court. Unlike homozygous *fru* mutants, *takeout* mutants did not display male-chaining behavior (data not shown), suggesting that these males are capable of distinguishing between males and females as potential mates. To verify that the mutation in *takeout*, rather than any other feature of the chromosome, is responsible for the reduced courtship, we introduced a P element carrying a wild-type genomic fragment encompassing the *takeout* transcription unit into this strain. When this transgene was crossed into the ry^{506} to^{1}/ry^{506} fru^{4} to^{1} genotype, normal levels of male courtship were restored (Fig. 7A). Due to the size and complexity of the *fru* gene, similar transgene rescue experiments with it were impractical; however, we found that introduction of a duplication of a chromosomal segment carrying the fru^{+} allele also restored normal courtship behavior to the ry^{506} to^{1}/ry^{506} fru^{4} to^{1} genotype (Fig. 7B). Taken together, these results confirm that a simultaneous reduction of *fruitless* and *takeout* function interferes with male courtship.

The original fru^{3} and fru^{4} mutant alleles were generated in a ry^{506} genetic background. Prior to the above studies, we noticed that the third chromosomes in our fru^{3} and fru^{4} strains carry not only these *fru* and *ry* mutations, but also the to^{1} mutant allele (data not shown). This led us to test whether previously observed courtship phenotypes associated with these *fru* alleles (Ryner et al. 1996; Villela et al. 1997) might have been enhanced by the presence of the *takeout* mutation. We compared the courtship of the double mutant flies (to^{1}, fru^{4}) with a recombinant fru^{4} line (to^{+}, fru^{4}) carrying the allele of *takeout* from the wild-type strain Canton-S (Fig. 6C). We again found that the presence of the *takeout* mutation caused a statistically significant reduction in courtship index. However, this effect was small in relation to that of the fru^{4} mutation alone.

The to^{1} strains used in all of the above analysis were maintained in homozygous condition for many generations before the discovery of the mutant allele. This led us to wonder whether selection for fertility had resulted in the accumulation of modifiers that suppress an effect of *takeout* on courtship behavior. We therefore outcrossed the to^{1} chromosome to wild-type (Canton-S) individuals for four generations, allowing free recombination between the Canton-S and *takeout* chromosomes (see Materials and Methods for details). When to^{1} homozygous mutants with the Canton-S background were recovered and tested for their ability to perform courtship behavior, a statistically significant reduction in courtship index was observed (Fig. 6D). Thus, *takeout* function is required for normal levels of male courtship in this background.

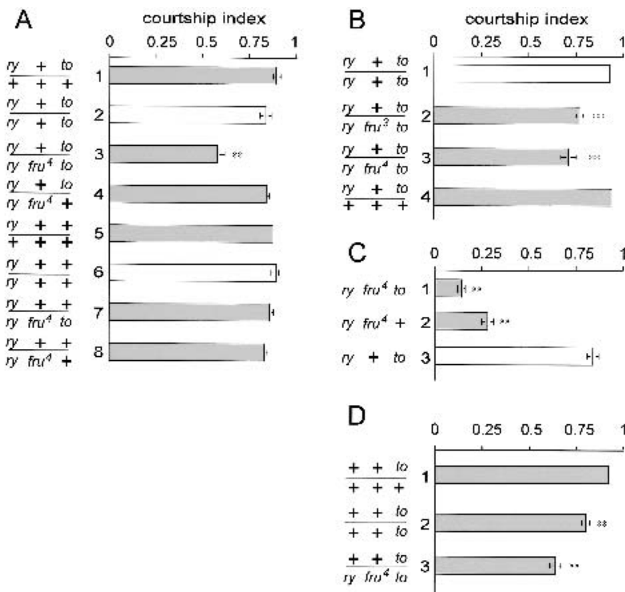

Figure 6. Mutations in *takeout* and *fruitless* interact to affect male courtship. Four sets of experiments are shown. (*A*) Courtship indices (± S.E.M.) of test males toward wild-type virgin females. The genotypes of males tested are indicated beside each bar. The different genotypes were generated by crossing various strains with ry^{506} to^1 (lanes *1–4*) or ry^{506} to^+ (lanes *5–8*). (Lane *1*) *takeout* heterozygous males; (lane *2*) homozygous mutant *takeout* males are not reduced in courtship; (lane *3*) males homozygous for *takeout* and heterozygous for fru^4 show a statistically significant reduction in courtship (marked by **, $p < 0.001$); (lane *4*) males heterozygous for both *takeout* and fru^4 show normal courtship. To control for a potential effect on courtship of the ry^{506} mutation on the *takeout* chromosome, parallel assays were performed on a ry^{506} strain that carries a wild-type *takeout* allele (to^+ry; lanes *5–8*). No effects of ry^{506} alone on courtship were observed. (*B*) Both the fru^3 and fru^4 allele interact with *takeout* to affect male courtship. *ry* phenotypes are indicated by white bars, ry^+ phenotypes by dark bars. The fru^4 and fru^3 mutations are caused by independent $P[ry^+]$ insertion. $n = 10$ for each genotype. (*C*) The courtship index of to^1 fru^4 double homozygous mutant males toward females is lower than that of fru^4 single mutant males alone ($n = 19$, $p < 0.001$). The courtship index of *takeout* single mutants is shown for comparison. (*D*) Males from a $takeout^1$ strain that was outcrossed to the wild-type Canton-S strain [to^1 (CS)] show a reduction in courtship ($n = 9$, $p < 0.005$).

Expression of takeout RNA is regulated by both Doublesex and Fruitless

We next examined how Dsx and Fru affect *takeout* expression. As *dsx* is known to affect sexual differentiation in males and females, it might either activate *takeout* in males, repress it in females, or both. Figure 8A shows *takeout* expression in *dsx* homozygous mutant animals compared with their heterozygous siblings. In repeated blot hybridization experiments, *XY dsx* individuals were found to have *takeout* RNA levels reduced by 37% relative to *XY dsx*/+ flies, indicating that the male-specific Dsx-M product functions to activate *takeout* expression. In chromosomally XX individuals, *dsx* mutations had an opposite effect. In comparison with *XX dsx*/+ siblings, *XX dsx/dsx* animals have levels of *takeout* mRNA increased by 13-fold, indicating that Dsx-F normally functions to repress *takeout* expression. Thus, the differential expression in males and females is achieved (at least in part) by *dsx*-dependent repression in females and activation in males. Curiously, *XX dsx/dsx* intersexes have more *takeout* RNA than do *XY dsx/dsx* intersexes (Fig. 8A), suggesting that sex-specific factors other than *dsx* also affect overall *takeout* expression.

We also examined the effect of the dominant dsx^{SWE} allele on *takeout* expression. Due to a deletion in the female-specific exon that results in constitutive male-specific splicing of the *dsx* pre-mRNA, this allele produces only Dsx-M (Nagoshi and Baker 1990). XY flies carrying this allele are phenotypically normal males, and did not have reduced *takeout* expression. However, in XX; dsx^{SWE}/+ animals, the presence of Dsx-M antagonizes Dsx-F function, resulting in intersexual flies that are similar in phenotype to those produced by *dsx* null mutations. We observed that *takeout* was derepressed to intermediate levels in such intersexes (Fig. 8B), further supporting the idea that *takeout* is controlled by *dsx*.

Analysis of *fru* mutants demonstrated that it affects *takeout* expression only in males. As shown in Figure 8C, repeated Northern analysis of RNA from *XY fru* adults showed that the levels of *takeout* RNA present in these individuals was consistently reduced by about 32% relative to *fru*/+ males. Expression of *takeout* was not increased by loss of *fru* function in *XX* females. This is consistent with the recent finding that functional sex-specific Fru protein is not present in females (Lee et al. 2000; Usui-Aoki et al. 2000). Taken together, the above

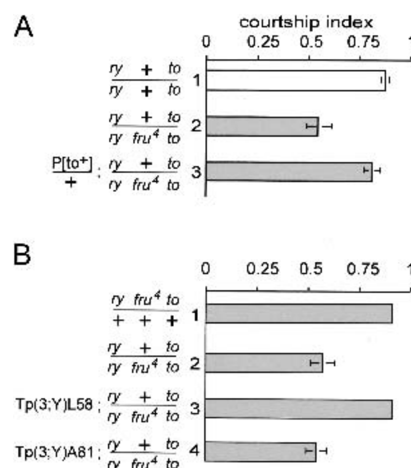

Figure 7. Rescue of the to^1/to^1, *fru*/+ courtship defect by wild-type *takeout* or *fruitless*. (*A*) A genomic $takeout^+$ transgene rescues the mutant phenotype ($n = 8$, $p < 0.05$). (*B*) Likewise, a duplication containing the wild-type *fruitless* gene (breakpoints at cytological locations 88D, 93D), rescues the courtship defect (lane *3*; $n = 6$, $p < 0.001$), whereas a control duplication of similar origin (breakpoints at cytological locations 75D, 80) does not (lane *4*; $n = 6$, $p < 0.001$).

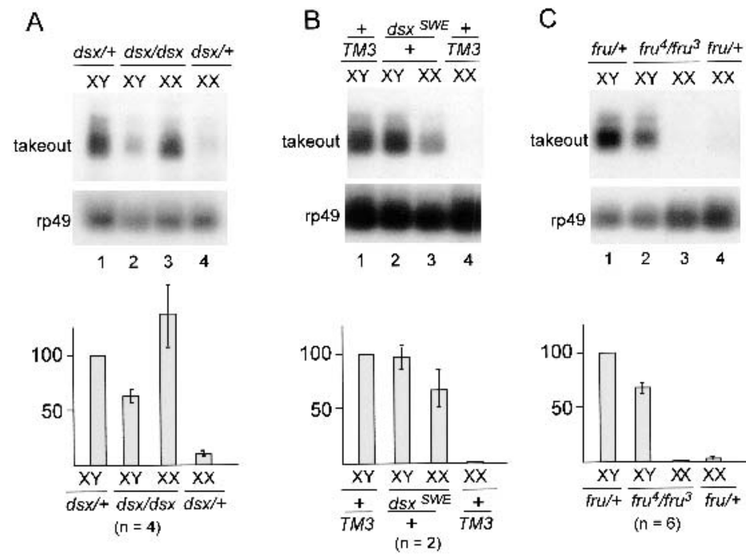

Figure 8. Expression of *takeout* is affected by both *doublesex* and *fruitless.* RNA from whole flies was analyzed. (*A*) Northern analysis of *takeout* expression in dsx^1 mutant flies shows that *takeout* expression in dsx^1 mutant males is reduced (lane *2*), and is derepressed in dsx^1 females (lane *3*) when compared with the expression in $dsx^1/^+$ siblings (lanes *1*,*4*). (*B*) Forced expression of male-specific forms of *dsx* induce *takeout* in XX individuals. Females expressing the male form of *dsx* from the dominant mutation dsx^{SWE} show activation of *takeout* (lane *3*) compared with control females (lane *4*). These males are dsx^{SWE}/dsx^+ and produce both Dsx-F and Dsx-M. There is no effect of dsx^{SWE}/dsx^+ on *takeout* expression in males (cf. lanes *1* and *2*). (*C*) *takeout* expression is reduced in fru^4/fru^3 males (lane *2*), but unaltered in fru^4/fru^3 females (lane *3*) compared with their heterozygous siblings (lanes *1*,*4*). (*Bottom*) Quantitation on the basis of several independent experiments (number indicated by n-value below each group). RNA levels were normalized to XY control males and rp49 controls.*takeout* expression in these males was assigned a value of 100 for each blot.

results indicate that both Fru and Dsx function to specify male-specific expression of *takeout* RNA.

Discussion

Differences in the morphology, physiology, and behavior of males and females undoubtedly reflects the sex-specific activities of numerous genes. Identification of these genes and analysis of their function is likely to lead to insights into the molecular events that underlie a variety of sex-specific processes affecting mating and reproduction. In *Drosophila*, the control of sexual differentiation is best understood in the soma, in which it is regulated by the combined action of the transcription factors Dsx and Fru, which are among the most terminal known components in the hierarchy. Functional analysis of these two factors has led to the recent suggestion that they have distinct and complementary roles, with Fru specifying sexual identity of tissues in the CNS that are responsible for courtship behavior, and Dsx specifying sex in other somatic tissues (Baker et al. 2001). However, given the observation that *dsx* mutants also have minor effects on courtship behavior (Villella and Hall 1996), we believe that a clear delineation of the roles played by Dsx and Fru will require more information about the specific genes and cell types whose sexual identity these factors specify.

Here, we have presented evidence that the *takeout* gene is a target of regulation by the somatic sex-determination pathway. Although *takeout* expression in some tissues is nonsex-specific, the vast majority of *takeout* RNA derives from fat body within the adult head and is specific to males. Surprisingly, our analysis of RNA from mutant flies indicates that sex-specific *takeout* expression depends on the function of both Dsx and Fru. Although this would seem to contradict the expected restriction of Fru function to the CNS, it is worth noting that the effect of Fru on *takeout* expression could be mediated indirectly by diffusible factors. In situ hybridization studies localized *fru* RNA to a variety of specific neurons (Ryner et al. 1996; Lee et al. 2000), but not the fat body cells in which male-specific *takeout* RNA is most prominently expressed. Interestingly, the only other instance of sexual differentiation outside of the CNS, where sex-specific Fru function is known to be required, is in the formation of the Muscle-of-Lawrence, a male-specific abdominal muscle in which sexual fate is determined through inductive signals that originate from the innervating motor neuron (Lawrence and Johnston 1986; Gailey et al. 1991; Usui-Aoki et al. 2000). In a previous study (So et al. 2000), circadian-regulated *takeout* RNA expression was found in several areas of the adult brain that were not detected in either our in situ hybridization studies or by reporters driven with the *takeout–GAL4* transgene, despite repeated attempts. Moreover, when this GAL4 construct was used to drive TraF-mediated feminization of males, virtually all endogenous takeout expression was eliminated, arguing that *takeout* is not normally expressed outside of the regions in which the driver is active.

Both the *dsx* and *fru* genes encode alternatively spliced transcripts that encode distinct forms of the Dsx and Fru proteins in males and females. Thus, both genes could potentially play a role in either activating *takeout* in males or repressing it in females. We found that full activation of *takeout* is not achieved in either *dsx* null or *fru* hypomorphic mutant XY individuals and, instead, *takeout* RNA is present at levels intermediate between those found in males and females. In chromosomal females, we found that only Dsx is required for repression of *takeout*. The fact that *fru* mutants do not affect *takeout* expression is consistent with experiments suggesting that the female-specific form of *fru* mRNA is not translated into a functional protein (Lee et al. 2000; Usui-Aoki et al. 2000). Moreover, all sex-specific Fru functions so far identified have been found in males (Gai-

ley and Hall 1989; Villella et al. 1997). Therefore, although a sex-specific Fru mRNA is produced in females that potentially encodes a protein, there is currently no evidence that it functions to regulate sexual differentiation.

The fact that *dsx* is capable of both activating and repressing *takeout* expression reflects the *dsx* gene's unusual ability to perform opposite functions in males and females by producing distinct proteins in the two sexes through alternative pre-mRNA splicing (Burtis and Baker 1989; Coschigano and Wensink 1993). The male-specific (Dsx-M) and the female-specific (Dsx-F) proteins share a common DM domain, which is required for DNA binding. The two proteins differ at their C termini, a region promoting dimerization in both forms (An et al. 1996; Erdman et al. 1996). The opposite activities of Dsx-M and Dsx-F at the molecular level have been most clearly shown in the case of the female-specific *yp2* gene, which like *takeout*, is expressed primarily in the adult fat body (Burtis et al. 1991; Coschigano and Wensink 1993; An and Wensink 1995). Both Dsx-F and Dsx-M bind the *yp2* promoter and affect its transcription in opposite ways. Bound Dsx-F activates *yp2* transcription, whereas bound Dsx-M represses its transcription. Thus, the effects of Dsx proteins on the *yp2* gene are reversed from those we observe on *takeout*. Three potential Dsx-binding sites (Erdman et al. 1996) are located within 1 kb upstream of the *takeout* translation initiation codon, but further studies will be required to determine whether Dsx proteins associate directly with the *takeout* promoter. Taken together, the results presented here and the findings from studies on yolk proteins suggest that Dsx-F and Dsx-M can each either activate or repress the activity of downstream genes. Presumably, the effect Dsx has on any particular gene is also determined by other regulators interacting with the gene's promoter. Consistent with this idea, Dsx-binding sites in the *yp2* promoter overlap those of other transcription factors necessary for tissue-specific activation/repression of *yp2* expression (An and Wensink 1995).

A possible role for Takeout and the fat body in mating behavior

The male-specific expression of its RNA in tissues closely associated with the adult brain suggested to us that secreted Takeout protein might affect male-specific behaviors that occur during courtship and mating. After outcrossing it from its original genetic background, we found that a *takeout* mutation reduced the ability of males to court and mate with wild-type females. Moreover, a significant synergistic effect on male courtship behavior was observed when to^1 was combined with either of two strong hypomorphic mutations in the *fru* gene to produce flies simultaneously reduced in both *takeout* and *fruitless* function. In *fru* heterozygotes, which have normal male courtship, reduction in *takeout* function caused a significant reduction in courtship index. Likewise, the effects of *fru* homozygous mutants on courtship were enhanced by reduction in *takeout* function. The fact that *takeout* only affects courtship in certain genetic backgrounds and the observed interaction of *takeout* and *fru* mutations, suggests the possibility that the *takeout* protein acts redundantly on courtship with genes under the control of *fru*. Redundantly functioning genes might include those from the *takeout* family itself, some of which we have found here to also be male-specifically expressed in adult heads. Consistent with this idea, the feminization of *takeout*-expressing cells in XY individuals using a *takeout* promoter driven Tra-F cDNA results in a reduction of courtship behavior of males toward females. This effect on behavior clearly exceeds that observed in *takeout* mutants, suggesting that other sex-specific factors involved in behavior are affected in the feminized tissues.

Studies on *Drosophila* sexual mosaics have identified a region in the posterior brain as the primary tissue in which male differentiation is required for courtship behavior (Hall 1979). However, these studies did not exclude the possibility that the male identity of other tissues are also necessary, and subsequent studies support this idea (Greenspan and Ferveur 2000). The effects on behavior that we observe when Tra-F is driven by a *takeout* promoter fragment suggest that tissues outside of the adult brain affect behavior. The *takeout–GAL4* transgene that we used to drive Tra-F was unable to produce detectable activity in the CNS. As these males were strongly reduced in courtship behavior, it seems likely that male differentiation is necessary in a *takeout*-expressing tissue outside of the CNS. It is worth noting here, however, that we cannot exclude the possibility that an undetectable level of TraF expression under control of the GAL4 driver in the CNS or in other cell types contributes to the observed effects.

Cells within the maxillary palp and third antennal segments in which the *takeout* promoter is active, could potentially mediate the perception of female pheromones. However, the requirement for these tissues in the courtship of females by males is unclear. The ablation of a large proportion of chemosensory sensillae in the antennae does not result in impaired courtship (Stocker and Gendre 1989), and other studies indicate that chemosensory organs on the proximal legs mediate the pheromonal response that stimulates courtship (Venard et al. 1989). Feminization of the antennal lobes, brain structures to which the antennal neurons project, leads to nondiscriminatory courtship of males toward both males and females, but does not lower courtship toward females (Ferveur et al. 1995; O'Dell et al. 1995). Current evidence for the involvement of maxillary palps in pheromone perception indicates that they mediate inhibitory rather than stimulatory effects on courtship (Stocker and Gendre 1989).

The only Takeout-expressing tissue in which there is clear evidence for sexual differentiation is the fat body surrounding the male brain, suggesting that it is responsible for the observed effect on courtship. How might fat body affect courtship behavior? These cells are an important source of products secreted into the hemolymph, which circulate throughout the adult body (Bownes and

Hames 1977; Haunerland 1996; Meister et al. 1997). The juvenile hormone-binding proteins from other insects, to which Takeout is related, are synthesized in the fat body and secreted into the hemolymph, where they are thought to carry juvenile hormone or other small lipophilic ligands to target cells (Nowock et al. 1975; Glinka et al. 1995; Wojtasek and Prestwich 1995). It is not known whether juvenile hormone plays any role in *Drosophila* male courtship behavior, but it has been shown that juvenile hormone stimulates the synthesis of accessory gland proteins (Herndon et al. 1997). A role for juvenile hormone has also been found in the reproductive behavior of the Caribbean fruit fly *Anastrepha suspense* (Teal et al. 2000). In this species, mating was found to be accompanied by an increase in the juvenile hormone levels of males and was correlated with competitive mating advantage of mated over unmated males. Topical exposure to juvenile hormone or related compounds caused virgin males from this species to release pheromones and to mate precociously, suggesting that juvenile hormone affects the propensity of males to initiate courtship behavior. If sex-specific proteins from the *takeout* family associate with juvenile hormone or other similar ligands, they could potentially exert sex-specific effects on the fly's physiology and behavior. There is already significant evidence that diffusible factors can affect reproductive abilities in *Drosophila*. For instance, *dissatisfaction*, a gene encoding a nuclear receptor has been shown to affect male and female reproductive behavior (Finley et al. 1998). Also, several proteins present in male seminal fluid have been shown to enter the female hemolymph and induce changes in oviposition as well as their receptivity to subsequent courting males (Monsma et al. 1990; Kubli 1992; Wolfner 1997; Lung and Wolfner 1999; Ottiger et al. 2000).

Starvation, mating, and circadian rhythms

In a previous study, *takeout* was shown to become induced in starving flies and to prolong their survival (Sarov-Blat et al. 2000). This raises the intriguing issue of how the starvation response might be related to mating behavior. One possibility is that *takeout* is involved in a mechanism governing how males expend their energy when faced with nutrient deprivation. It is easy to imagine that pathways exist for managing the choice between foraging and courtship behavior that are critical for the male's survival and reproductive success. The observation that, in addition to sex and nutrition, *takeout* also responds to circadian rhythms (Sarov-Blat et al. 2000; So et al. 2000; Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Lin et al. 2002) suggests that it integrates a variety of signals that affect the adult male's behavior.

Materials and methods

RNA subtraction

Poly(A$^+$) RNA from isolated heads was prepared as described (Chandler et al. 2001). To create *tra-2* null flies, we used two overlapping deficiencies, *tra-2*^{PM6} and *tra-2*^{PM7} (McGuffin et al. 1998). RNA was isolated from XX; *tra-2*^{PM6}/*tra-2*^{PM7}, which are transformed into males, and from their *tra-2*^{PM6}/*CyO* or *tra-2*^{PM7}/*CyO* heterozygous female siblings. The Clontech PCR-select cDNA subtraction kit (catalog no. K1804-1) was used for subtraction. PCR fragments were subcloned, and inserts with preferential expression in males were identified by Northern analysis as described (Mattox et al. 1996). The inserts from clones of interest were used to probe an adult head cDNA library (Palazzolo et al. 1990; Hamilton et al. 1991), a gift from P. Hardin (University of Houston, TX).

Fly strains

Flies were kept on standard cornmeal/sugar-based food at 25°C under noncontrolled light conditions. The *fru*^{4}/*TM3,Sb,ry* and *fru*^{3}/*TM3,Sb,ry* strains were a gift from B. Baker (Stanford University, Stanford, CA). The *takeout* mutant used was identified in Bloomington stock number 2541 (*sn*^{w}; *ry*^{506}). The UAS–traF strain (stock no. 4590) and the *to*$^+$, *ry*^{506} control strain (stock no. 225), as well as the *Tp(3;Y)L58*, *y*$^+$/*TM6,Ubx; C(1)RM*, *y*^{1}/*C(1;Y)1*, *y*^{1} (Baker 1980; stock no. 2914) and *Tp(3;Y)A81*, *y*$^+$/*TM6,Ubx; C(1)RM*, *y*^{1}/*C(1;Y)1*, *y*^{1} (stock no. 2787) strains were obtained from the Bloomington stock center. The genotypes of other strains used were as follows: *w*^{1118}/*B*^{S}*Y*; *p*^{p} *dsx*^{1}/*TM3, Sb* and *w*^{1118}/*B*^{S}*Y*; *dsx*^{SWE}/*Df(dsx)*/*TM3, Sb*.

Takeout^1 outcrossed flies were generated by backcrossing of the original *sn*^{w}; *ry*^{506} *to*^{1} strain to *Canton-S* for four generations. Individual chromosomes were isolated over a *TM3* balancer of similar background and the presence of *to*^{1} was verified by PCR using a single nucleotide polymorphism in the coding region.

Generation of recombinant chromosomes

The *to* mutant alleles present in the *fru*^{3}, *fru*^{4}, and *dsx*^{1} chromosomes were replaced with the wild-type allele from Canton-S by meiotic recombination. Balanced males from lines carrying potential recombinant chromosomes were tested by PCR for the presence of the P[PZ] transposon insertion associated with both of the *fru* mutations (Castrillon et al. 1993). The *takeout* mutant and *to*^{CS} alleles were identified in amplified products using a single nucleotide polymorphism in the coding region. The presence of the *to*^{CS} allele was verified by observing expression of *takeout* RNA on Northern blots.

RNA Blot hybridizations and RT–PCR

DNAse-treated RNA from *Canton-S* flies was reverse transcribed using the Superscript II kit (GIBCO) with oligo(dT)$_{12–18}$ as primer. Amplifications of *takeout* and rp49-derived cDNAs were performed simultaneously in 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.3 at 25°C), 0.1% Triton X-100, 1.5 mM magnesium chloride, 0.6 µM primer (each), 0.1 mM dNTPs, and 5 units of Taq polymerase (Roche). Amplifications were carried out by first incubating for 2 min at 94°C, then 17 cycles of 30 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C, followed by 10 min at 72°C. Half of the reaction was analyzed by Southern hybridization using end-labeled oligonucleotides. RNA equivalent to 15 isolated antennae and 0.6 µg RNA from whole flies was used per PCR reaction.

Hybridizations were done at 42°C in 1 M sodium chloride, 0.4% polyvinyl pyrollidone, 0.4% Ficoll, 0.4% BSA, 0.1 M PIPES (pH 7.0), 0.2% SDS, and 100 µg of salmon sperm DNA/mL. Blots were washed twice in 6× standard saline citrate (SSC) at 25°C for 10 min, then once in 6× SSC at 50°C for 20 min. The

PCR primers used were as follows: ACCTTCTCACTCGTTG GAC and AATGTTTTTATGGTATTTTACAGA for *takeout*; AGCATACAGGCCCAAGA and GTGTATTCCGACCAGGTT for *rp49*. The oligonucleotides used as ^{32}P-labeled probes for hybridization were GTTGTCACCCAGCGCCTTGT for *takeout* and AGCATACAGGCCCAAGA for *rp49*. For Northern hybridizations of various takeout family members, probes were generated by amplification of genomic DNA using the primers described as follows: CG11852, (1)GAACGATGGAGCGGC AACA, (2)GTTCCTCAACGACAACTGGAC; CG2016, (1)CG AGTCGCGTGAAAGTTG, (2)ACGGAAGTTGGCTAAGATGG; CG1124, (1)GCCTTAATCCCTTAGATTGT, (2)CACAGGGT GAATACGACTCT; CG5867, (1)CGCATTTGCATCGGTCAG GT, (2)CTATTTCCGCTGCCAAACATAAGC; CG7096, (1)CC TTGCAACGATCCGATGAT, (2)CAAGTAGAGCTTTCAAC AAAACC.

Northern blots were analyzed by phosphorimaging and quantitation using Kodak 1D 3.5 image quantitation software.

In situ hybridization to fly sections/X-gal staining

In situ hybridizations to frozen serial sections were performed using a modified version of the protocol of Han et al. (1992). In short, fresh flies were frozen in a fly collar. The 10-µm frozen sections were cut on a Leitz cryostat, dried at room temperature for 30 min, fixed in 4% paraformaldehyde in PBS for 10 min, and then washed in PBS. The sections were deacetylated, washed in PBS, and prehybridized in a humid box for 2 h at 60°C. Hybridizations were performed overnight at 60°C. Prehybridization and hybdridization buffer was 5× SSC, 50% formamide, 5× Denhardt's, 250 µg/mL yeast tRNA, 500 µg/mL salm sperm DNA, 50 µg/mL heparin, 2.5 mM EDTA, 0.1% Tween 20, 0.25% CHABS, and 0.1% SDS. Slides were washed at 65°C in 2× SSC/ 50% formamide 2 × 30 min., 2× SSC 2 × 15 min., 0.2× SSC 3 × 20 min., eqilibrated in PBS + 0.1%Triton X-100 (PBX) 1× 10 min., and blocked for 1 h at room temperature with 10% goat serum in PBX. They were incubated in the same buffer with a 1:1000 dilution of anti-DIG antibody (Roche) at 4°C overnight, washed 3 × 30 min in PBX, and stained following the supplier's protocol (Genius kit, Roche), including 1 mM levamisole in the reaction buffer.

For X-gal staining, 10-µm frozen sections were cut on a Leitz cryostat, dried at room temperature for 5 min, fixed in 1% glutaraldehyde in PBS for 10 min, and washed in PBS. X-gal staining was done as described by Fischer and Maniatis (1988). Sections comparing males and females were prepared and stained together on the same slide.

Probes for takeout

Northern analysis of *takeout* expression was performed with a 250-bp fragment from the 3′ untranslated region of the *takeout* gene as a probe. Fragments were labeled with ^{32}P by random priming (Roche) or by PCR following the protocol of Mertz and Rashtchian (1994). The primers used to obtain the fragment were CTTCAAAGTGGGCAGAGTC and AATGTTTTTATG GTATTTTACAGA. Probes from a full-length cDNA clone were also used with identical results. All *dsx* and *fru* mutant strains used for RNA preparations carried recombinant chromosomes in which the Canton-S allele of *takeout* was introduced.

For in situ hybridizations, the same 250-bp PCR fragment from the 3′ end of the gene was subcloned and used as a template to generate DIG-labeled riboprobes (Roche). Identical results were also obtained with a nonoverlapping 383 nucleotide probe from the N-terminal part of the *takeout* protein-coding region defined by the primers 5′-TAAGTATGGTGATGGC GAATGTAT-3′ and 5′-TACCTTGCCCTGGATGTTATAG-3′.

takeout–Gal4 *transgenic flies*

At 1.27 kb immediately upstream of the *takeout* translation, start codon were amplified by PCR (primers, 5′-ATAAGAAT GCGGCCGCTGCTAACACGTCTATAACT-3′ and 5′-ATAAG AATGCGGCCGCACTGGTTCTGCTTCTGCGG-3′) and cloned into the *Not*I site of *pCaSpeR4-GATN*. The construct was verified by sequencing. pCaSpeR4-GATN was made by subcloning the Gal4 coding region of *pGATN* (Brand and Perrimon 1993) as a *Kpn*I/*Spe*I fragment into *pCaSpeR4* (Pirrotta 1988). Transgenic lines were established in a w^{1118} background.

Courtship assays

Males were collected within 2–4 h of emergence under light CO_2 anesthesia, and individually stored in food vials for 4–6 d. For observation of courtship behavior, individual males were aspirated without anesthesia into a chamber (diameter 0.8 cm) of a plastic mating wheel, together with a 2–4-h-old Canton-S virgin female. The courtship index was calculated as the fraction of time the male spent displaying any element of courtship behavior (orienting, following, wing extension, licking, attempted copulation, copulation) within a 10-min observation period (Taylor et al. 1994).

Short-term activity assay

Assays were performed as described by Anand et al. (2001). Males were collected within 2–4 h of emergence under light CO_2 anesthesia, and were individually stored in food vials for 4–6 d. Individual males were aspirated without anesthesia into a chamber (diameter 0.8 cm) of a plastic mating wheel containing a filter paper with a single line dividing the chamber in half. After 2–3 min of acclimation time, the number of times the male crossed the center line within the 3-min observation time was counted.

Takeout *and* fruitless *rescue experiments*

The 6.4-kb genomic *takeout* rescue fragment, obtained from P1 phage DS02779 DNA by restriction digests with *Xho*I and *Eco*RV, was sucloned into the Carnegie20 transformation vector. Transgenic strains were established in sn^w; $ry^{506}to^1$ flies. Heterozygous transformants were crossed to obtain externally identical siblings both with and without the transgene. Following the courtship assay, genomic DNA was prepared individually from each tested male (Gloor and Engels 1992) and the presence of the $takeout^+$ transgene assessed by PCR.

Males carrying a duplication of the *fruitless* gene were produced by crossing *Tp(3;Y)L58*, $y^+/to^1/TM6$, *Ubx*; *C(1)RM*, y^1 females to y^1; to^1, $fru^4/TM3$, *Sb* males. Control flies were generated by crossing of *Tp(3;Y)L58*, $y^+/to^1/TM6$, *Ubx*; *C(1)RM*, y^1 females to Canton-S males and by crossing of to^1, $fru^4/TM3$, *Sb* females to to^1 males. Control males carrying a duplication unrelated to the *fruitless* locus (*Tp(3;Y)A81*) but with similar origin, were produced in the same way.

Statistical analysis

Analyses were performed with JMP 2.0 statistical software (SAS Institute, Inc.). Following initial analysis of variance (ANOVA),

comparisons between groups were by unplanned multiple comparisons using Tukey-Kramer analysis at $\alpha = 0.05$.

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