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Control of Male Sexual Behavior and Sexual Orientation in Drosophila by the *fruitless* Gene

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Summary

Sexual orientation and courtship behavior in Drosophila are regulated by *fruitless (fru)*, the first gene in a branch of the sex-determination hierarchy functioning specifically in the central nervous system (CNS). The phenotypes of new *fru* mutants encompass nearly all aspects of male sexual behavior. Alternative splicing of *fru* transcripts produces sex-specific proteins belonging to the BTB-ZF family of transcriptional regulators. The sex-specific *fru* products are produced in only about 500 of the 10^5 neurons that comprise the CNS. The properties of neurons expressing these *fru* products suggest that *fru* specifies the fates or activities of neurons that carry out higher order control functions to elicit and coordinate the activities comprising male courtship behavior.

Introduction

Sexual reproduction in species ranging from fruit flies to humans is preceded by elaborate courtship behaviors. In Drosophila courtship, the male engages in a series of actions including orienting toward and following the female, tapping her with his forelegs, singing a species-specific courtship song by extending one of his wings and vibrating it, licking the genitalia of the female, and curling his abdomen to attempt copulation (reviewed by Hall, 1994; Greenspan, 1995). The behavior of the female largely consists of running away. However, if she has not recently mated and is sufficiently stimulated by the courtship of the male, she will slow down, open her vaginal plates, and allow copulation (reviewed by Hall, 1994).

Like other aspects of sexual differentiation, sexual behavior is governed by a hierarchy of sex-determining regulatory genes (reviewed by Burtis, 1993; McKeown, 1994). In brief, the presence of two X chromosomes in diploid individuals initiates female development, with the activation of the *Sex-lethal (Sxl)* gene. *Sxl* autoregulates the splicing of its own pre-mRNA and activates *transformer (tra)* by regulating splicing of the pre-mRNA of *tra*. The products of the *tra* and the *transformer-2 (tra-2)* genes then regulate the splicing of the pre-mRNA of the *doublesex (dsx)* gene, leading to the production of a female-specific Dsx protein. In males, where *Sxl* and *tra* do not make functional products, *dsx* pre-mRNA is spliced in its default pattern, resulting in a male-specific Dsx protein that differs from the female form at its carboxy-terminus. The Dsx proteins function as sex-specific transcription factors that regulate many aspects of sexual differentiation, including the external morphologies of both sexes and aspects of their internal biochemistry.

The *Sxl*, *tra*, and *tra-2* genes control all aspects of somatic sexual differentiation (reviewed by Burtis, 1993). However, *dsx* does not control all aspects of somatic sexual differentiation. For example, *tra* and *tra-2*, but not *dsx*, control the development of a male-specific abdominal muscle called the Muscle of Lawrence (MOL; Taylor, 1992). In addition, constitutive expression of the male form of the Dsx protein transforms females into morphologically wild-type males, but they do not court (Taylor et al., 1994). These results suggest that there is a previously unrecognized branch (or branches) in the sex-determination hierarchy just downstream of *tra* and *tra-2* that controls MOL development and many aspects of male sexual behavior. Interestingly, mosaic analysis suggests that the development of the MOL depends on the sex of the neurons that innervate it (Lawrence and Johnston, 1986).

Since both sexual behavior and MOL development depend on nervous system function, the new branch of the sex determination hierarchy is expected to operate there. Regions of the central nervous system (CNS) of the fly have been identified that are important for the performance of specific steps of male courtship (reviewed by Greenspan, 1995), but almost nothing is known about either the neuronal circuitry that underlies courtship behavior or the developmental processes required to organize these circuits. In addition, altered sexual orientation has been correlated with both the feminization of specific brain regions (reviewed by Greenspan, 1995) and the misexpression of the *white* gene in the brain (Zhang and Odenwald, 1995; Hing and Carlson, 1996).

Although many genes affect courtship performance of Drosophila males, *fruitless (fru)* is one of the very few that appears to be specific to male courtship (reviewed by Hall, 1994; Taylor et al., 1994). In *fru* mutant males, the later steps of courtship, from singing through copulation, are abnormal or absent. Because *fru* mutant males fail to copulate, they are sterile. In addition, *fru* males court both males and females indiscriminately. When *fru* mutant males are grouped together, they form male–male courtship chains in which each male is both courting and being courted. An additional *fru* phenotype

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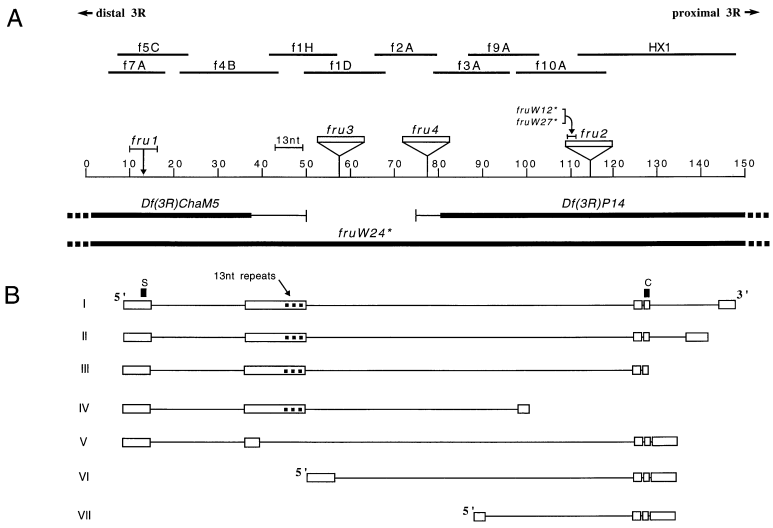

Figure 1. Genomic Region Encompassing the *fru* Transcription Unit

(A) Lines at top of drawing represent overlapping DNA clones that span approximately 140 kb of the *fru* gene. Positions of the fru^1, fru^{w12}, and fru^{w27} chromosomal breakpoints are indicated by arrows; the fru^2, fru^3, and fru^4 P element inserts are indicated by inverted triangles; the bar shows uncertainty in the position of the 13 nt repeats. Deletions of the region are indicated by heavy black lines. Asterisks indicate new mutants.

(B) Organization of the *fru* transcription unit. Open boxes represent genomic fragments that hybridize to *fru* cDNAs. (Each of these boxes may represent more than one exon.) Boxes connected by solid lines represent fragments from the same class of transcripts. The regions used to generate probes for in situ hybridization are indicated by black boxes above the cDNA structures: C, common coding region; S, specific to transcripts that produce the sex-specific RNAs of *fru*. Transcripts of classes I–V are shown to extend to the distal-most fragment; however, only two cDNA clones extended this far. All the other cDNAs of classes I–V were shorter at their 5′ ends but were truncated at different positions relative to each other.

is that the male-specific MOL is incompletely formed or absent (Gailey et al., 1991). There are no phenotypic effects of *fru* in females (reviewed by Hall, 1994). Thus, *fru* is a promising candidate for a gene functioning in the proposed new branch of the hierarchy.

Here, we report the cloning and characterization of the *fru* gene through two convergent lines of research, one designed to identify the gene at the top of the proposed new branch of the hierarchy, the second a chromosomal walk through the *fru* gene. At the time this paper was about to be submitted, a paper appeared that reported cloning part of the *fru* gene (Ito et al., 1996).

Results

Molecular Cloning of *fruitless*

To identify the gene at the top of the proposed new branch of the sex-determination hierarchy, we looked for other genes containing the target site through which Tra and Tra-2 regulated *dsx* pre-mRNA splicing, a 13 nt sequence that is repeated six times in *dsx* (reviewed by McKeown, 1994). A synthetic oligonucleotide probe containing three copies of the 13 nt *dsx* repeat sequence was used to probe a genomic library. Other than DNA from the *dsx* locus, the strongest hybridizing genomic DNA was a 645 bp EcoRI fragment found in eight overlapping DNA clones. This fragment contained a cluster of three copies of the 13 nt *dsx* repeat (data not shown) and hybridized to salivary chromosome position 91B, where the *fru* gene resides (Gailey et al., 1991).

We independently cloned *fru* using two P element insertion alleles, fru^3 and fru^4 (Castrillon et al., 1993), as starting points for a chromosomal walk that spans approximately 180 kb, from the *glass (gl)* gene located proximal to *fru* to the proximal breakpoint of a deficiency *(Df(3R)148.5-1)* located distal to *fru* (Figure 1A; see Experimental Procedures). Other *fru* alleles mapped across 110 kb of the walk (Figure 1A). The 645 bp EcoRI fragment containing the 13 nt repeats hybridized approximately 12 kb distal of fru^3 (Figure 1A), confirming that this fragment came from *fru*.

fru Produces Both Sex-Specific and Sex-Nonspecific Transcripts

Analysis of 35 cDNAs shows that the *fru* transcription unit spans at least 140 kb and produces at least seven overlapping classes of transcripts (Figure 1B). The presence of three different 5′ ends suggests that there are at least three promoters. Most transcripts contain a common middle region. Additional complexity comes from the presence of at least four alternative 3′ ends. It is not known whether transcripts with a particular 5′ end can have all the possible 3′ ends and vice versa. Intriguingly, transcripts generated from the most distal promoter appear to be alternatively spliced near the 13 nt repeats: part of the repeats-containing region is missing in the cDNA of class V (Figure 1B).

Northern analysis of poly(A)+ RNA from wild-type adult male and female heads (very little signal was detected in poly(A)+ RNA from bodies) revealed a complex set of *fru* transcripts, some of which were sex-specific. When a probe with sequences common to all known classes of cDNAs was used (see Experimental Procedures), the strongest signals detected in adult head mRNA were three female-specific (9.0, 8.0, and 7.4 kb), three male-specific (7.9, 6.4, and 5.4 kb) and one sex-nonspecific transcript (4.4 kb; Figure 2A).

A Subset of *fru* Transcripts Is Sex-Specifically Spliced under the Control of *tra* and *tra-2*

DNA sequencing revealed that the alternatively spliced transcripts from the distal promoter were generated by use of 5′ splice sites 1,590 nt apart (Figure 2B). To determine whether this 5′ splice site selection was sex-specific, we carried out reverse transcription–polymerase

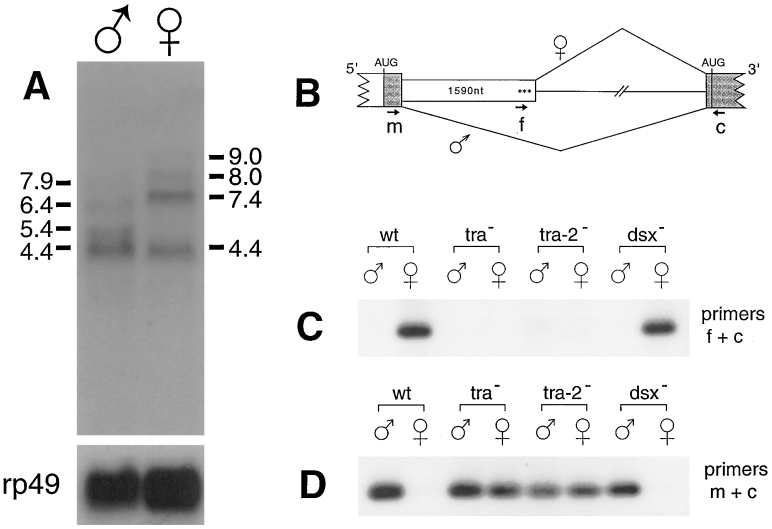

Figure 2. *fru* Produces Multiple Transcripts, Including Sex-Specific Transcripts That Are Alternatively Spliced in a *tra*- and *tra-2*–Dependent Manner

(A) Northern blot analysis of poly(A)+ mRNA isolated from sexed wild-type (wt) adult heads. Top: *fru* transcripts detected by hybridization with a probe made from the BTB coding portion of the *fru* cDNAs. Approximate sizes in kb of detected *fru* transcripts are indicated. Bottom: the same blot hybridized with a probe from the ribosomal-protein gene *rp49*, shown as an RNA-loading control.

(B–D) Sex-specific alternative splicing of *fru* transcripts in *tra*, *tra-2*, and *dsx* mutants.

(B) Schematic drawing of the alternative 5′ splice sites of *fru* that are joined to a common 3′ exon. Exons are indicated by rectangles; jagged sides indicate that only a portion of the exon is shown; thin lines represent the introns. Positions of primers m, f, and c used for RT–PCR are indicated by short lines with arrowheads. Shaded regions have protein coding potential.

(C and D) Southern analysis of RT–PCR products generated using the primers indicated on the right of each panel and total RNA from sexed adult heads with the genotypes: wt, wild-type Canton S; *tra*$^{-}$, *tra*^{1}*/Df(3L)st*; *tra-2*$^{-}$, *tra-2/Df(2R)trix*; and *dsx*$^{-}$, *In(3R)dsx23/Df(3R)dsx15*, as indicated above each lane.

chain reaction (RT–PCR) followed by Southern analysis and DNA sequencing (Figure 2B). Such analysis of the downstream 5′ splice site revealed a *fru*-specific RT–PCR product in RNA from wild-type female, but not male, heads, indicating that this splice site is used only in females (Figure 2C). Similar analysis of the upstream 5′ splice site revealed that the transcript class missing the repeats region was only detected in males (Figure 2D). Thus, the upstream 5′ splice site is used only in males and the downstream 5′ splice site is used only in females.

RT–PCR was also performed on RNA isolated from chromosomally male (XY) and female (XX) flies carrying mutations in *tra* or *tra-2*. In XX; *tra/tra* (and XX; *tra-2/tra-2*) flies, the male form of *fru* transcripts was produced, while the *fru* splicing pattern was unaffected in XY flies mutant for either *tra* or *tra-2* (Figures 2C and 2D). These results demonstrate that sex-specific splicing of *fru* transcripts is indeed controlled by *tra* and *tra-2*. Interestingly, the mechanism of this regulation (5′ splice site choice) appears to differ from the one used to control *dsx* pre-mRNA splicing (activation of a 3′ splice site). These findings establish that *fru* is part of the somatic sex-determination hierarchy and likely represents the gene at the top of a newly discovered branch of this hierarchy. Consistent with this conclusion, the sex-specific splicing of *fru* transcripts was unaffected by *dsx* (Figures 2C and 2D).

The hypothesis that *fru* is controlled by *tra* and *tra-2* was also tested genetically. If *fru* is controlled by *tra* and *tra-2*, then XX flies doubly mutant for *fru* and *tra* (or *tra-2*) should exhibit the *fru* MOL and behavioral phenotypes. We used the *fru*^{1} mutation because *fru*^{1} mutants exhibit high levels of male–male chaining and have an MOL distinct from the wild-type male MOL fibers, as well as the normal female MOL–homologous fibers (Gailey et al., 1991; Taylor and Knittel, 1995). The XX; *tra fru* and XX; *tra-2*; *fru* double mutants have an abnormal MOL muscle like that of *fru*^{1} mutant males (Figure 3A) and groups of XX; *tra fru1* flies chained vigorously, in a *fru*-like manner (chaining index, 41 ± 11, n = 5; wild-type males chaining index, 0, n = 4 ; see Experimental Procedures). These anatomical and behavioral results are consistent with the function of *fru* being downstream of *tra* and *tra-2*.

fru Has Homology to the BTB-ZF Family of Transcription Factors

DNA sequencing reveals that the male-specific form of *fru* transcript class I contains a long open reading frame that starts upstream of the male-specific 5′ splice site and encodes a polypeptide of 776 amino acids (Figure 4). In females, use of the female-specific 5′ splice site introduces a stop codon into the open reading frame at residue number 94. However, use of a start codon downstream of the female-specific 5′ splice site would result in a polypeptide of 675 amino acids in the same reading frame as the male form, but missing its first 101 amino acids. The 101 amino acids specific to the male form contain a stretch of 12 histidines alternating with neutral residues, followed by a proline-rich stretch (Figure 4). The functional significance of these motifs is currently unknown, but several other Drosophila transcriptional regulators contain a similar histidine repeat.

Both the male and long female open reading frames encode proteins containing a BTB domain (also called a POZ domain), a domain of approximately 115 amino acids, suggested to function in dimerization (reviewed by Albagli et al., 1995). The *fru* BTB domain is followed by stretches of repeated amino acids, including runs of alanines and asparagines, glutamate alternating with arginine or aspartate, and stretches rich in glycine, glutamate, and serine. The carboxy-terminus of the protein contains a zinc finger pair related to the C_2H_2 class. Our sequence data, not presented here, show that at least

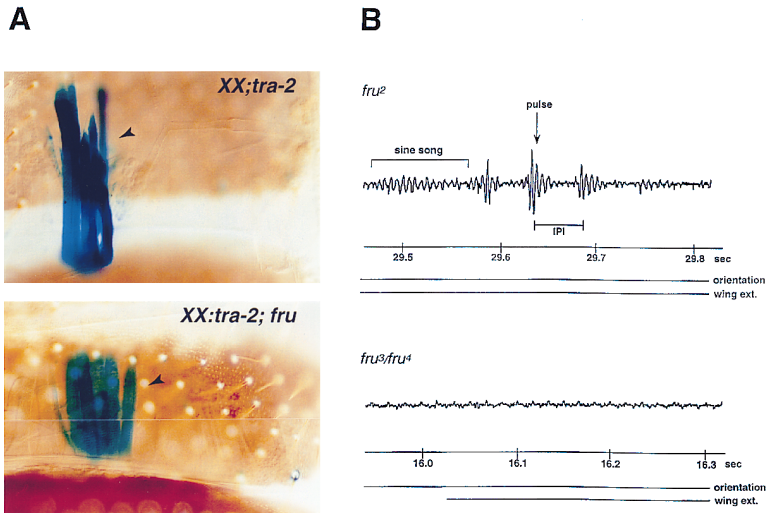

Figure 3. Phenotypes of *fru* Mutants: The MOL and Courtship Song

(A) *fru* and *tra* or *tra-2* double mutants have a *fru* MOL phenotype. MOL fibers (arrowheads) of abdominal segment 5 stained for β-galactosidase. (Top) a normal-appearing male-like MOL (about 280 μm in length) in an XX; *tra-2/tra-2*;P{79B actin ry^+ *lacZ*} *ry* fru^1/+ adult. (Bottom) a characteristic fru^1-defective MOL (about 150 μm in length), in an XX; *tra-2/tra-2; P[79Bactin-lacZ, ry^+] ry fru1*/P{79B actin ry^+ *lacZ*} *ry* fru^1 adult. The following genotypes were assayed for the size of the MOL, and all had an abnormal MOL phenotype similar to that in fru^1 homozygotes (some not shown): *st tra* fru^1/*st tra* fru^1, (XX, N = 26; XY, N = 24); *tra-2/tra-2*; fru^1/fru^1, (XX, N = 11); *tra-2/Df(2L)trix*; fru^1/fru^1, (XX, N = 6); *tra-2B/tra-2B*; fru^1/fru^1, (XX, N = 16); *tra-2B/Df(2L)trix*; fru^1/fru^1, (XX, N = 11), where N = number of MOLs examined.

(B) Combination song-recording/courtship-behavioral traces from homozygous fru^2 and heterozygous fru^3/fru^4 males in the presence of a Canton-S (wild-type) virgin female. Each trace represents only a fraction of a second, taken from 5–8 min recordings obtained for a given male. The time scales shown are in real time extracted from the taped recordings in seconds (sec). Orientation and wing extensions are shown as two separate lines below each acoustical trace. IPI, interpulse interval. fru^3/fru^3, fru^3/*Df(3R)Cha*^{M5}, and fru^3/*Df(3R)P14* males were also recorded for a total of 400 min of courtship (data not shown), and no pulse-song or sine-song bouts appeared in the audio records obtained for any of these flies in male–female and male–male experiments (aggregate n = 79 males recorded); during 5.5 of these 400 min, the wing of a given male was extended. The corresponding values for fru^4 (same kinds of genotypes and experimental situations as just indicated) were 486 min total recording time and 9.7 min of wing extension time (n = 92). In contrast, wild-type (Canton-S) males were previously reported to generate 338 pulses per min and 20 sine-song bouts per min (Villella and Hall, 1996).

two other alternative *fru* transcripts encode proteins with different zinc finger pairs at their carboxy-termini.

Proteins with the same overall structure as Fru have been termed the BTB-ZF protein family (Hu et al., 1995a) or Ttk subgroup (Albagli et al., 1995). A set of two family members, Ttk and BR-C, have been shown to be sequence-specific transcription factors (Read and Manley, 1992; von Kalm et al., 1994). Both contain alternative carboxy-terminal zinc finger pairs that confer distinct DNA-binding specificities and are expressed in overlapping, but different, cell-specific patterns (Read et al., 1992; Emery et al., 1994; von Kalm et al., 1994). Thus, these proteins may regulate different target genes in a cell-specific manner.

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MMATSQDYFGNPYALFRGPPTTLRPRESPLGVGHPHGHGHLHSHAHAHGHGHAHSHYAALDLQT
PHKRNIETDVRAPPPPLPPPPLPLPPASPRYNTDQGAMDQQFCLRWNNHPTNLTGVLTSLLQRE
ALCDVTLACEGETVKAHQTILSACSPYFETIFLQNQHPHPTIYLKDVRYSEMRSLLDFMYKGEV
NVGQSSLPMFLKTAESLQVRGLTDNNNLNYRSDCDKLRDSAASSPTGRGPSNYTGGLGGAGGVA
DAMRESRDSLRSRCERDLRDELTQRSSSSMSERSSAAAAAAAAAAAVAAAGGNVNAAAVALGLT
TPTAAAAAAVAAAVAAAANRSASADGCSDRGSERGTLERTDSRDDLLQLDYSNKDNNNSNSSST
GGNNNNNNNNNNNNSSSNNNNSSSNRERNNSGERERERERERERDRDRELSTTPVEQLSSSKRRR
KNSSSNCDNSLSSSHQDRHYPQDSQANFKSSPVPKTGGSTSESEDAGGRHDSPLSMTTSVHLGG
GGGNVGAASALSGLSQSLSIKQELMDAQQQQQHREHHVALPPDYLPSAALKLHAEDMSTLLTQH
ALQAADARDEHNDAKQLQLDQTDNIDGSSARHHLSTPLSTSSSASPPPPPFGMHLSAALKREYH
PLHYMAAGNGHNGPSALGYGNQGSGNAPNSAGGAGSVAGGVGAGGGAGGATGAAGHNSHHTMSY
HNMFTPSRDPGTMWRCRSCGKEVTNRWHFHSHTAQRSMCPYCPATYSRIDTLRSHLRVKHPDR
LLKLNSSI
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Figure 4. Polypeptide Sequence Derived from Class I *fru* cDNAs

The amino-terminal methionine begins the peptide encoded by the male form of the *fru* transcript, and the asterisk indicates the potential start methionine for the peptide encoded by the female form of the transcript. Arrow indicates divergence point for alternative carboxy-termini. Shaded region encodes the BTB domain. Amino-terminal histidine repeat and carboxy-terminal zinc finger pairs are enclosed in open boxes. Proline-rich stretch near amino-terminal and cysteine and histidine pairs of the zinc fingers are indicated by underline.

fru Controls a Range of Male-Specific Nervous System Functions and Is Essential in Both Sexes

None of the published *fru* alleles interrupt coding sequences (see Figure 1A), suggesting that they may not be nulls. Indeed, RT–PCR detects *fru* transcripts from every mutant and mutant combination (data not shown). A set of three new *fru* alleles is described here (see Experimental Procedures). fru^{w24} is a small deficiency that spans the *fru* locus (Figure 1A). fru^{w12} and fru^{w27} are a translocation and an inversion, respectively, each of which has one break in the fru^2 insert and the other distant from the *fru* locus (Figure 1A). Strikingly, the fru^{w12} or fru^{w27} mutations were lethal in both sexes, in combination with the deficiency fru^{w24} and with each other, indicating that *fru* encodes a vital function. Transcripts produced from the two proximal *fru* promoters were expressed sex-nonspecifically (data not shown) and are thus likely to be responsible for the vital function of *fru*. The nature of the lethal defect is currently not known. RT–PCR performed using mRNA isolated from the lethal combinations, fru^{w12}/fru^{w24}, fru^{w27}/fru^{w24}, and fru^{w12}/fru^{w27}, at a stage well before their late pupal lethal phase, detected *fru* transcripts when primers to the common coding region were used, suggesting that transcription was occurring from heterologous promoters introduced by the rearrangements or that an undetected

Table 1. Courtship Behavior of *fru* Mutants

Genotype	male + male			male + female		
	All courtship CI* (%)	Wing ext. (%)	N	All courtship CI* (%)	Wing ext. (%)	N
wild-type	4 ± 1	0 ± 0	10	84 ± 3	46 ± 7	7
fru^1/fru^1	51 ± 4	21 ± 3	25	61 ± 5	35 ± 4	21
fru^3/fru^3	32 ± 5	1 ± 0	31	15 ± 5	0 ± 0	20
fru^4/fru^4	41 ± 6	2 ± 1	25	29 ± 7	2 ± 1	20
fru^3/fru^4	42 ± 7	1 ± 0	16	22 ± 8	1 ± 1	13
fru^3/fru^{w12}	1 ± 0	0 ± 0	9	1 ± 1	0 ± 0	10
fru^4/fru^{w12}	8 ± 5	1 ± 1	9	7 ± 5	0 ± 0	12
fru^{w12}/Cha^{M5}	2 ± 1	0 ± 0	14	0 ± 0	0 ± 0	6
fru^{w27}/Cha^{M5}	2 ± 2	0 ± 0	15	0 ± 0	0 ± 0	7

* CI, courtship index.

proximal *fru* promoter was still in place (data not shown). However, fru^{w12}/fru^{w24} produced barely detectable levels of *fru* transcripts as compared with wild type. These mutant combinations represent the strongest *fru* loss-of-function mutations available and demonstrate that *fru* has a sex-nonspecific vital function.

These new *fru* mutations and fru^3 and fru^4 (Castrillon et al., 1993) were assayed for their effects on male sexual behavior, MOL development, and locomotion (see Experimental Procedures). When tested in pairs in courtship chambers, fru^3, fru^4, and fru^3/fru^4 males, like fru^1, courted indiscriminately (Table 1), failed to copulate, and had MOL defects. In addition, fru^3, fru^4, and fru^3/fru^4 males showed very little wing extension (Table 1) and during these wing displays generated no song pulse signals (see Figure 3B). This defect is specific to courtship, as these mutants are normal for flight (see Experimental Procedures) and are able to flick their wings when rejecting advances made by another male. Further reductions in courtship were found with fru^{w12} in combination with fru^3 or fru^4 and with $Df(3R)Cha^{M5}$ in combination with fru^{w12} or fru^{w27}. These males barely courted at all (Table 1), yet they exhibited essentially normal locomotor activity (see Experimental Procedures). All mutant combinations did show some male–male chaining, although chaining was reduced in the strongest mutant combinations (data not shown). Thus, the early steps of courtship (orientation, following, and wing extension) as well as the later steps (courtship song and attempted copulation) are disrupted in these mutants and mutant combinations.

The new phenotypic effects of these *fru* mutations extends the wild-type *fru* function to all, or nearly all, steps of courtship behavior. Since the most extreme mutant combinations examined performed little sexual behavior beyond male–male chaining, and since RT–PCR indicated detectable levels of sex-specifically spliced transcripts, it is possible that a null for the sex-specific function of *fru* would show no sexual behavior.

fru Is Expressed in a Distinct Pattern in the Adult CNS

The array of cells expressing sex-specific transcripts of *fru* was determined by in situ hybridization using a probe corresponding to the 5′ common exons from the most distal *fru* promoter (probe S; see Figure 1B). This probe should only detect transcripts of the male form in males and the female form in females, owing to the tight regulation of the sex-specific splicing of *fru* (see above). In both sexes, only about 500 of the roughly 10^5 neurons of the CNS were labeled; there was no labeling in other tissues of the body. In both sexes, labeled neurons were found most commonly as small groups and less frequently as single cells, and most labeled cells were distributed in similar areas of the brain and ventral nerve cord of males and females (Figures 5A–5D). A set of nine groups of labeled neurons, ranging from 10–30 cells, was detected in the CNS of males in positions likely to be involved in particular male courtship behaviors (see below). Comparably positioned cells were found in six of these locations in females.

A set of two groups of labeled neurons was detected in areas that must have a male identity for flies to perform particular steps of male courtship (reviewed by Greenspan, 1995). One was a prominently labeled group of cells in the dorsal–posterior protocerebral region (Figure 5E); early steps in courtship (following and wing extension) were mapped to this region using gynandromorphs. The second was a group of cells labeled in males but not in females (Figure 5B) that is located in the ventral mesothoracic ganglion, where courtship song has been mapped.

fru-positive neurons were also found in areas not known to be involved in sexual behavior but nevertheless associated with higher order nervous system functions that could be involved in courtship. For example, three groups of neurons in the anterior ventrolateral protocerebrum were found associated with higher order sensory processing neuropils of the CNS (Heisenberg, 1994; data not shown), and there was a male-specific neuronal cluster in the abdominal ganglion, a ganglion that controls abdominal movements during copulation (Figure 5F).

We also note that the cell bodies of many of the neurons expressing the sex-specific transcripts of *fru* were small (approximately 5 μm in diameter) and compactly clustered (e.g., Figure 5B), suggesting that these cells may function as local circuit interneurons, which would be involved in the production of complex patterned motor activity and modulating reflexes (e.g., Burrows, 1992). In addition, there were a small number of isolated

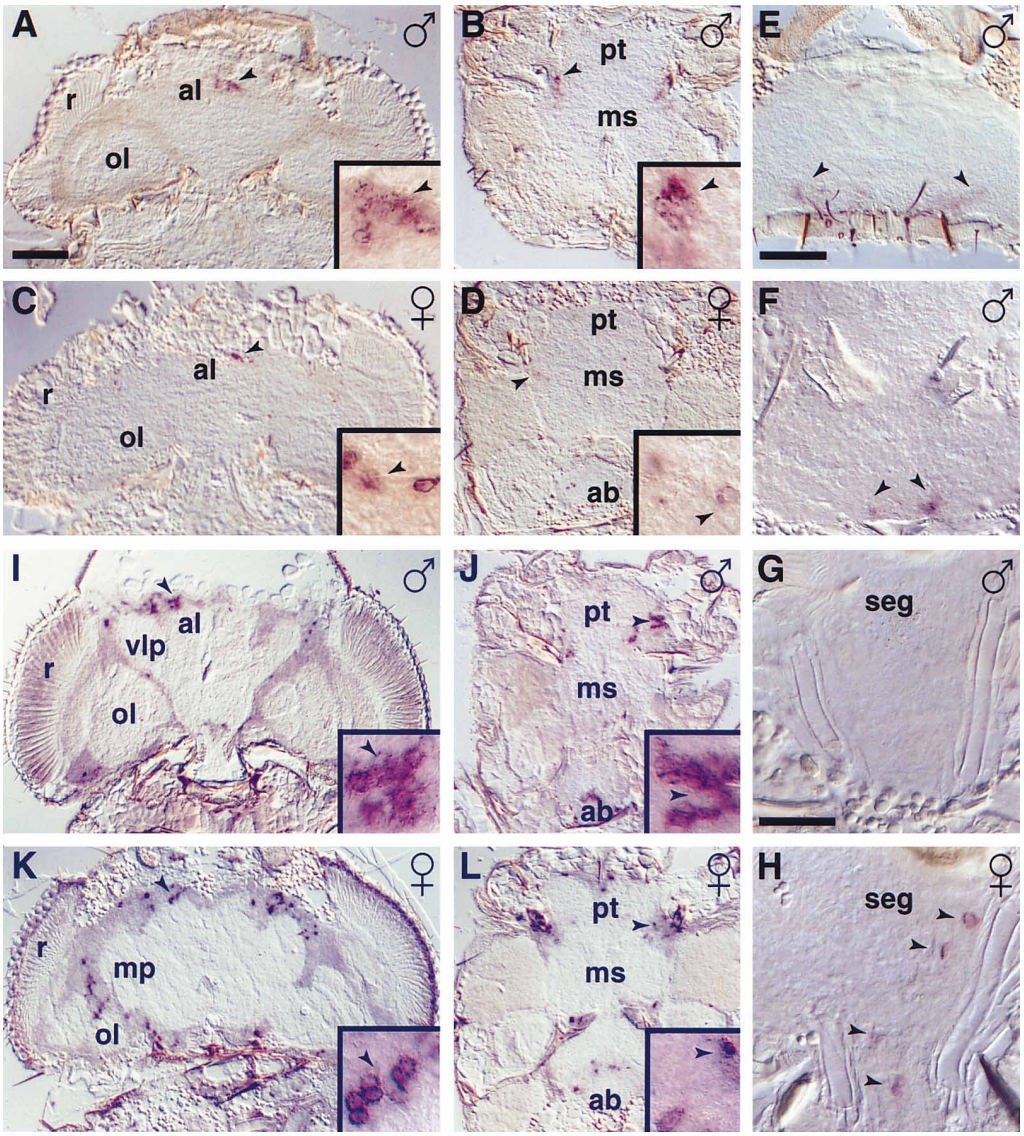

Figure 5. Expression Pattern of *fru* Transcripts in Horizontal Sections of Pharate Adult Male and Female Flies

(A–H) Sections labeled with probe S and (I–L) sections labeled with probe C (see Figure 1A). Scale bar shown in (A) (also for [B], [C], [D], [I], [J], [K], and [L]) equals 100 μm. The inserts for these figures have a width of 25 μm. Scale bar shown in (E) (also for [F]) equals 100 μm. Scale bar in (G) (also for [H]) equals 40 μm. For all sections (A–L), top of panel is anterior and bottom posterior.

(A) Section through a male head with antennal lobe (al) showing a bilateral cluster of medial antennal lobe neurons (arrow and insert).

(B) Section though a male thorax showing laterally unstained thoracic musculature and along the midline, the ventral nerve cord with *fru*-labeled bilaterally symmetric male-specific clusters of about 30 neurons (arrow and insert) located between the prothoracic (pt) and mesothoracic (ms) ganglia. The abdominal ganglion does not appear in this particular plane of section.

(C) Section through a female head at the level of the antennal lobe and ventrolateral protocerebrum; same as in (A).

(D) Section through a female thorax with a few labeled neurons (arrows) in the mesothoracic ganglion. Insert, same region as in (B).

(E) Labeled neurons in the dorsal brain (arrows).

(F) Labeled bilaterally symmetric cluster of abdominal neurons found in males but not in females (see also [D] and [L]).

(G and H) Sections through the subesophageal ganglion (seg) of a male and female brain.

(G) No neurons are labeled in the male ganglion. (H) A set of four neurons is visible in the female ganglion (arrows).

(I) Section through male head at the level of the antennal lobe and ventro-lateral protocerebrum (vlp) showing several groups of heavily stained neurons (arrow and insert).

(J) Section through male thorax showing heavily labeled clusters of neurons between the prothoracic and mesothoracic ganglion (arrow and insert same as in [B]) and in the abdominal ganglion at the same position as those stained in (F).

(K) Section through a female head at a slightly more dorsal section than the male in (I), showing several clusters of dorsal anterior neurons (arrow and insert).

(L) Section of a female thorax. Only a few labeled neurons are present (arrow and insert), not the clusters of labeled cells as found in the male shown in (J) and (B). Sections in (C), (I), (J), and (K) are not orthogonal, so bilaterally symmetric labeled neurons appear only on one side.

neurons of larger size expressing the sex-specific transcripts of *fru*; these could be either interganglionic interneurons making connections throughout the brain and ventral nerve cord, or motor neurons.

The small number and the locations of the neurons expressing male-specific transcripts of *fru* suggest that *fru* is directly involved in only some of the sensory and motor systems necessary for male sexual behavior. For example, the sex-specific transcripts of *fru* are abundantly expressed in a group of primary sensory interneurons in the antennal lobe (Figure 5A) involved in the processing of chemosensory information (Stocker, 1994). Based on the medial–anterior locations of their cell bodies, these cells appear to be relay interneurons known to project to higher brain centers such as the calyx of the mushroom body (Figures 5A and 5C; B. J. T. et al., unpublished data; Stocker, 1994). However, *fru*-positive cells are not present in the primary interneurons of the lamina and medulla involved in processing visual information; yet vision plays an important role in male courtship (reviewed by Hall, 1994). Similarly, few, if any, of the motor neurons that innervate direct or indirect flight or leg muscles express the sex-specific transcripts of *fru*, yet muscular activity is required for courtship song production and locomotion.

One unexpected aspect of the expression pattern of *fru* was that some *fru*-expressing cells were detected only in females; for example, in the subesophageal ganglion (Figures 5G and 5H), yet no female behavioral phenotypes of extant *fru* mutations have been detected (see Hall, 1994). Although it is possible that the female-specific *fru* mRNA is not translated, it seems more likely that there are *fru*-related female phenotypes more subtle than could have been detected by the assays done to date.

The fact that some of the neurons expressing transcripts from the distal *fru* promoter were sex-specific suggests the involvement of other regulatory processes. This additional sex-specificity does not appear to be provided either by transcripts produced by alternative splicing events at the 3′ end of the transcript or by use of the other two promoters, because these do not appear to be sex-specifically regulated (data not shown). Likely origins of this additional control are transcriptional regulation of the distal promoter or sex-specific cell death or neurogenesis.

The intensity of hybridization also differed between the labeled neurons in male versus female CNSs. Neurons in female CNSs showed high levels of cytoplasmic hybridization compared with the faint level of hybridization seen in male neurons (see insert, Figure 5C). Male neurons, however, showed strong nuclear labeling detected as dark dots (see insert, Figure 5A). These findings are perhaps indicative of a slower processing rate of the primary transcript or transcripts in males with subsequently lower steady-state levels of mature cytoplasmic transcript. The female transcript may be processed faster due to the activities of Tra and Tra-2.

The expression pattern of RNAs from all *fru* promoters was determined by hybridization to sections of wild-type pharate adults with a probe common to most (6 of 7) transcript classes (probe C; Figure 1B). Most or all neurons in the CNS, as well as a number of nonneuronal cells, expressed *fru* (Figures 5I–5L). Within the nervous system, cells exhibited a wide range of staining intensities. Almost all neurons had a low level of rather uniform staining; a small subset of neurons was moderately to heavily stained in the CNS of both sexes. The expression pattern of the more heavily labeled cells overlapped with the staining detected with the 5′ upstream exon probe (see above), suggesting that these heavily labeled neurons may primarily contain transcripts derived from the distal promoter. Other tissues labeled by the common coding-region probe were a subset of direct flight muscle cells, nurse cells, spermatocytes, fat body cells, gut epithelium, and ectodermal epithelium, including the internal and external genitalia (data not shown). The level of expression in nonneuronal tissues was weaker than that of the intensely stained neurons in the CNS.

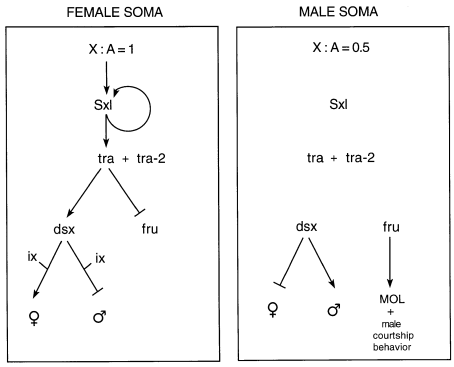

Figure 6. Schematic of the Somatic Sex-Determination Regulatory Hierarchy Including the *fru* Branch

X:A, the ratio of X chromosomes relative to sets of autosomes. *ix*, *intersex* gene. Arrows indicate activation of gene function, and "hammerheads" indicate repression of gene function.

Discussion

On the Genetic Control of Sexual Behavior

Our findings validate the genetic inferences (Taylor, 1992; Taylor et al., 1994) that there is a branch in the Drosophila sex-determination hierarchy controlling male courtship behavior, sexual orientation, and the development of a male-specific muscle. The fact that *fru* expression is controlled by *tra* and *tra-2* firmly establishes *fru* as residing in such a branch of the hierarchy (Figure 6). It is likely that *fru* is at the top of this new branch, directly under the control of *tra* and *tra-2*, because *tra* and *tra-2* regulate a sex-specific splice choice in the *fru* pre-mRNA in a region that contains three copies of the 13 nt Tra/Tra-2 binding sites.

Insight into the aspects of sex controlled by the *fru* branch of the hierarchy comes from the phenotypes of *fru* mutations (reviewed by Hall, 1994; Taylor et al., 1994). Our behavioral analysis of new *fru* mutations extends the known wild-type *fru* functions to encompass essentially all aspects of male courtship.

The similarities between the *fru* proteins and other BTB-ZF family members suggest that the *fru* protein isoforms function as transcription factors with multiple DNA-binding specificities and cell-specific expression

patterns. The fact that *fru* probably encodes transcription factors raises the intriguing possibility that *fru*, like *dsx*, is the final regulatory gene in its branch of the hierarchy. If so, *fru* would directly control the expression of downstream genes responsible for governing sex-specific MOL development, sexual orientation, and the behaviors that comprise male courtship.

The *fru* sex-determination branch functions in roughly 0.5% of the cells of the CNS and not in other tissues. The neurons expressing the sex-specific *fru* transcripts are not only found in regions of the CNS in which the cells responsible for particular steps of male courtship behavior reside (reviewed by Greenspan, 1995) but are also associated with higher order neuropils (Heisenberg, 1994). Our findings suggest that the male-specific function of *fru* is to specify the fates, or activities, of neurons that carry out integrative or command functions, or both, to elicit and coordinate the complex array of activities that comprise male courtship behavior.

Sexual Dimorphism in CNS and Sexual Behavior

It is clear that in many species, the CNS plays a major role in the control of sexual behavior, but this understanding rests largely on studies of either physiological or morphologically detectable sexual dimorphisms. The sexual dimorphisms most commonly described are neurons unique to one sex and nuclei (identifiable groups of neuronal cell bodies) that differ in cell number between the sexes; sexual dimorphisms have also been described as neurons present in both sexes that differ in size, shape, anatomical connections, or physiology (e.g. Breedlove, 1992; Heisenberg, 1994; LeVay, 1996). In many cases, such dimorphisms have been shown to be intimately associated with the performance of sex-specific behaviors. For example, several sexually dimorphic nuclei in the hypothalamus have been shown to be involved in the generation of male or female sexual behaviors (reviewed by Breedlove, 1994; Swaab and Hofman, 1995).

With regard to Drosophila, one surprising aspect of our findings is that there are relatively few differences in the patterns of cells expressing the sex-specific transcripts of *fru* in the two sexes. Assuming that in males most cells expressing these transcripts are involved in the control of sexual behaviors, these results imply that much of male sexual behavior may be governed by neurons for which there appear to be corresponding cells in females. This does not mean that comparably positioned cells in the two sexes are identical. Rather, we expect that further characterization of these cells will reveal differences in their projection patterns or physiology or both, most obviously because they express different Fru proteins that are likely transcription factors regulating different aspects of cell function.

Developmental Aspects of the Establishment of Sexual Behavior

The role of *fru* in specifying male sexual behavior could be to direct neurogenesis or differentiation or both. Although our experiments do not distinguish among these possibilities, experiments using temperature-sensitive alleles of *tra-2 (tra-2^{ts})* have provided insights (Belote and Baker, 1987). Adult females display male courtship behavior only if wild-type *tra-2*, and presumably therefore male-specific *fru* activity, is absent during the latter part of the pupal period. Since cell division in the CNS ceases at an earlier point in development, the primary function of the male-specific products of *fru* is most likely to control differentiation.

Additional experiments with *tra-2^{ts}* mutations suggest that expression of the male-specific *fru* products in adult females may be sufficient to induce male sexual behavior. In these experiments, XX *tra-2^{ts}* individuals were reared to adulthood at the permissive temperature, at which they developed into slightly intersexual female adults who displayed no male sexual behaviors, and then shifted to the restrictive temperature (Belote and Baker, 1987). Such a shift, which should prevent subsequent *tra-2* function and by inference allow synthesis of the male-specific *fru* products to begin, led to these individuals acquiring many aspects of male courtship behavior after a period of some days. This finding suggests the possibility that the sex-determination hierarchy must function continuously, at least in females, to maintain the appropriate state of sexual differentiation in the CNS. However, since the XX *tra-2^{ts}* individuals reared at the permissive temperature are morphologically somewhat intersexual, it is not clear whether this inference applies just to these females or also applies to wild type. Nevertheless, these observations do raise the possibility that "female" cells might redifferentiate into the male state, and there is in fact some postnatal neuronal plasticity in flies (Heisenberg et al., 1995).

With regard to the finding that *fru* functions prior to adulthood to establish sexual behavior, it is interesting to note that in some vertebrates (e.g., rats and monkeys) the levels and distributions of steroid sex hormones (androgens and estrogens) play important roles in a variety of sexual behaviors, including mating behavior, and that these hormones have their most dramatic effects during early development (reviewed by Breedlove, 1994; Swaab and Hofman, 1995). These hormones are proposed to affect the organization of the developing brain such that it is predisposed to particular sexual behaviors during adulthood. The possibility that androgens might have similar roles in humans in organizing the brain with regard to sexual behaviors is suggested by studies of females affected by congenital adrenal hyperplasia, which leads to abnormally high fetal androgen levels (reviewed by Breedlove, 1994; Swaab and Hofman, 1995).

Genes and Sexual Orientation

The finding that *fru* mutations lead to males courting females and males indiscriminately, whereas wild-type Drosophila melanogaster males normally recognize only females as appropriate courtship objects, implies that one function of *fru^{+}* is to provide males with the ability to discriminate females from males as appropriate individuals to court. Thus, sexual orientation in flies is controlled by the same hierarchy of genes that controls all other aspects of sex.

Whether these results are directly relevant to other species, in particular mammals, is currently unclear.

While there is still relatively little molecular information on the genes controlling sexual differentiation in mammals, it appears that mammals and flies use unrelated sex-determination mechanisms (Ryner and Swain, 1995). However, human and mouse homologs of the *tra-2* gene have been recently isolated, although their functions in vertebrates are unknown (Matsuo et al., 1995; Banfi et al., 1996; Dauwalder et al., 1996; Segade et al., 1996).

In considering more generally the question of what role genes play in determining sexual orientation, one must first recognize that species differ widely in the relative contributions of genes and environment to male sexual behavior. Male courtship behavior (and, by implication from our results, also sexual orientation), in flies is a "fixed action pattern", i.e., is largely genetically programmed, although courtship behavior in flies can be modified to a limited degree by experience (reviewed by Hall, 1994; Greenspan, 1995). At perhaps the other extreme, human male courtship behavior seems to be highly modifiable by experience (our unpublished data). Nevertheless, there is a variety of evidence in vertebrates, including humans, suggesting that male sexual behavior, including sexual orientation, has a genetic component (reviewed by Breedlove, 1994; LeVay, 1996). First, we note evidence that a sexually dimorphic portion of the human brain (known in mammals to be involved in courtship and mating behavior) is also dimorphic in homo- versus heterosexual males (reviewed by LeVay, 1996). Second, prenatal exposure to sex steroid hormones affects the types of sexual behaviors displayed by mature mammals, and hormone and receptor levels are under genetic control. Third, the choice of male targets by homosexual rams appears to have a heritable component that involves a steroid hormone axis (Perkins et al., 1995). Fourth, the possibility that sexual orientation could have a genetic etiology in humans is also suggested by results from twin studies on male homosexuals (reviewed by LeVay, 1996) and by a mapping experiment involving familial occurrences of homosexuality in males (reviewed by LeVay, 1996; see also Hu et al., 1995b).

Experimental Procedures

13 nt Repeats Screen

Genomic clones that hybridized to a single-stranded DNA probe (3 × repeats probe: 5′-GATCCATCTTCAATCAACATAGATCCATCT TCAATCAACATAGATCCATCTTCAATCAACATAGATCCACTAGTT CTAGA-3′) were isolated from eight genome equivalents of a genomic library (Maniatis et al., 1978) by plaque hybridization (Sambrook et al., 1989), except that hybridization was done without formamide at 42°C and washes were at 40°C in 6 × SSC, 0.5% SDS. DNAs from 42 positive phage clones were isolated, digested with EcoRI, and tested for cross-hybridization to each other and to a *dsx* clone (nucleotide coordinates: 2513–3053; Burtis and Baker, 1989) by Southern analysis. A set of 12 groups of non-*dsx* clones was found, 4 of which also cross-hybridized with a probe that contained a portion of the *dsx* gene that includes five of the six 13 nt repeats of *dsx* (coordinates: 2793–3053). The clone showing the strongest hybridization with both probes contained the 645 bp EcoRI fragment. The 645 bp EcoRI fragment was subcloned into Bluescript pSK(+) phagemid vector (Stratagene) and sequenced using a United States Biochemical "Sequenase 2.0" kit (GenBank accession number U72491). In situ hybridization to polytene chromosome squashes used a genomic clone with an approximately 15 kb genomic insert containing the 645 bp EcoRI repeats-containing fragment.

Molecular Cloning

A bidirectional chromosomal walk was carried out using as starting points genomic DNA fragments that flank the fru^3 and fru^4 insertion sites (isolated by plasmid rescue [Pirrotta, 1986]) until the fru^2 insertion site and the fru^1 breakpoint were identified. The positions of the *fru* mutants were determined by Southern analysis and in situ hybridization to salivary gland chromosomes in the case of the fru^1 inversion and the *Df(3R)P14* and $Df(3R)Cha^{M5}$ deficiency breakpoints. Clones that overlapped the fru^2 insertion location and extended proximally to the *glass* gene were a gift from K. Moses (cosmid HX1, l phage 3-10 and 5F2) and The Genome Mapping Project (Institute of Molecular Biology and Biotechnology, Greece; cosmid clones: 15A5, 47H3, 18D7; data not shown). A set of three clones that overlapped the fru^1 inversion break and extended distally was isolated from a cosmid library (Tamkun et al., 1992) by screening with a subcloned fragment from phage f7A (Figure 1A) and extended the walk 15–25 kb to the proximal breakpoint of *Df(3R)148.5-1* (data not shown).

cDNA Isolation and Characterization

3′ rapid amplification of cDNA ends–PCR was used to generate a partial *fru* cDNA clone (3′ RACE System, GIBCO BRL). Gene-specific primers were as follows: fru-1 = 5′-GACGTGTGACGATGGAGC AAC-3′, fru-2 = 5′-CGATCCAGATCGAAAGAGAATATCATCA-3′. An approximately 450 bp partial *fru* cDNA was isolated by this method. Sequences from the 3′ end of the partial cDNA were used to generate a new *fru*-specific PCR primer, fru-4-rev = 5′-AGGCGTGATCATTAT GATATTGTAGCAA-3′, which was used in combination with the fru-1 and fru-2 primers to screen for the presence of *fru* cDNA clones in DNA isolated from cultures of five different cDNA libraries. By this method, a λZAP adult head library (DiAntonio et al., 1993) was found to contain *fru* cDNAs. Plating and screening of this library, using the approximately 450 bp PCR product and the 645 bp fragment as probes, resulted in the identification of nine *fru*-specific cDNAs. An additional 26 cDNAs were isolated upon further screening of the library (approximately 1 million plaques screened) using the largest of the cDNAs isolated from the first screen as a probe. Restriction maps and Southern analysis identified overlaps between the different cDNAs and grouped them into classes (I–VII). Representative cDNAs were used to identify XhoI–SalI restriction fragments of the genomic walk (or EcoRI–HindIII fragments in the case of cosmid HX1) from which the cDNAs were derived.

The nucleotide sequences of cDNAs of class I and the 5′ end of class V were determined as described above (GenBank accession number U72492). Comparison of the sequences of class V cDNA, class I cDNA, and the 645 bp fragment showed that the alternative 5′ splice sites were at coordinates 469 and 2059. PCRs of Drosophila genomic DNA and the class I cDNA were compared to show that the sequence between the two alternative 5′ splice sites contained no additional introns, by using overlapping primer sets that amplify products that cover the region. Homology searches against available databases used the BLASTP program (Altschul et al., 1990).

Northern Analysis

Total RNA from Canton-S wild-type adult heads and bodies was isolated using TRIzol Reagent (GIBCO BRL). Adult heads were separated from bodies and appendages by sieving in liquid nitrogen. Poly(A)+ RNA was purified by using the PolyATract mRNA Isolation System (Promega). Poly(A)+ RNA (5 μg) was separated on formaldehyde/agarose gels and transferred to a Hybond-N+ membrane as described by the manufacturer (Amersham). Hybridization was done with a PCR–derived BTB domain probe (*fru* coordinates: 2075–2422). The *rp49* transcript was detected as an RNA-loading control by reprobing the blots (O'Connell and Rosbash, 1984).

RT–PCR

The 496 bp female-specific and 322 bp male-specific RT–PCR products were generated with a commercial kit (SuperScript Preamplification System for First Strand cDNA Synthesis, GIBCO BRL) using total adult head RNA (5 μg) and random primers. PCRs used the following gene-specific primers: Primer f: 5′-GGGAATTCGAGGACG TGTGACGAT-3′ Primer m: 5′-TGCATTACGCGGCCTTGGACTT-3′ Primer c: 5′-GGAAATCGTCTCGAAGTAGGAC-3′. PCR products

were analyzed by Southern analysis (Sambrook et al., 1989). Each PCR product was sequenced by cycle sequencing on an ABI 373A sequencer (Applied Biosystems, Inc.).

To assay for the presence of *fru* transcripts in the genotypes fru^{w24}/fru^{w12}, fru^{w24}/fru^{w27}, and fru^{w12}/fru^{w27}, we isolated RNA from 1–3 day-old pupae (10–12 pupae each) as above. RT was performed on total RNA from an equivalent of two pupae for a given genotype using primer fru-8-rev (5′-GTGAGACCACGCACCTGCAG-3′). PCRs were performed on one-tenth of the RT reactions using the primer set fru-25 (5′-AACACTGACCAAGGACGATG-3′) and fru-26-rev (5′-ATG GGCAGCGAACTCTGGCC-3′). Southern analysis was used to confirm that the PCR products were bona fide *fru* products.

Mutagenesis

New rearrangements at *fru* (of which fru^{w24}, fru^{w12}, and fru^{w27} are described here) were induced on a fru^{2} chromosome by X-ray mutagenesis (A. A. et al., unpublished data). These chromosomes were back-crossed three generations to a *w, DrPr/TM3, Sb ry* stock before making balanced stocks. Chromosomal break-points of these aberrations are as follows: fru^{w24} = Df(3R)$gl^{-}fru^{-}$, *91A1; 91D2-3,* fru^{w27} = In(3R)88C; 91B1-2 + In(3LR)68A-B; 92E and fru^{w12} = In(3LR)65C-D; 91B1-2 +T(3, het.)91B1-2, het. ("het." equals a breakpoint in salivary-gland heterochromatin). All three mutations are homozygous-lethal. Their genomic break-points were mapped to a 2.8 kb BamHI–HindIII fragment at the 5′ end of *white* gene of the P(w^{+}, ry^{-}) insert.

The survival rates of heteroallelic males and females were as follows: fru^{w24}/fru^{w12} = 0% (163), fru^{w24}/fru^{w27} = 0% (187), and fru^{w12}/fru^{w27} = 2% (375). The numbers of heterozygous control siblings are in parentheses.

Behavioral Analyses

Behavioral assays and song recordings were carried out on males aged individually for 7–14 days then placed in a small chamber with either another male of the same genotype or with a wild-type virgin female (1–5 days old). Simultaneous video and audio recordings were done for bouts of 5–8 min (Taylor et al., 1994; Villella and Hall, 1996). The courtship index values (reviewed by Hall, 1994) were measured as the percentage of the observation periods that a given test male courted the other male or the female. Courtship included tapping, orientation, following, wing extension, and attempted copulation. The percentages of time during which wing extensions occurred were measured separately. For male–male tests, each courtship index is the percent of time male number 1 (the first male to initiate courtship for more than 20 s) courted male number 2. (Only one male had his behavior quantified because the first male to initiate courtship tends to be the dominant courter, with the other male being largely the courtee.)

To assay sterility, we placed single males (age 5–10 days) with 3–4 wild-type females (3–5 days old), and the absence of larva was scored 7–10 days later.

The chaining index of groups of eight males each were determined by 10 min observations. The proportion of the observation period during which three or more males courted one another in a conga line or other kind of cluster was quantified using timers. Males were aged individually for 5–6 days, then grouped together in a food vial (day 0), and observations were made on day 3 or 4.

Gross flight tests and flight wing-beat recordings were performed as described previously (Kulkarni and Hall, 1987; Barnes et al., submitted). Short- and long-term locomotor activity were assayed as in Kulkarni and Hall (1987) and Hamblen-Coyle et al. (1989), respectively. Most of the mutant types in Table 1 were tested and gave flight or locomotor values in the normal range (data not shown).

MOL Analysis

Muscle fibers on dissected dorsal cuticle spanning the fifth abdominal segment were examined under polarized light and were identified as belonging to the MOL by the expression of β-galactosidase produced from a P{79B actin ry^{+} *lacZ*} element insert as in Taylor and Knittel (1995). The abdominal musculature was examined by polarized light in the *fru* mutants fru^{3} (n = 11), fru^{4} (n = 16), and fru^{3}/fru^{4} (n = 8), and no MOL was detected.

In Situ Expression Analysis

Pharate adult Canton-S flies were frozen and sectioned on a cryostat at 20 μm and prepared for in situ hybridization as described elsewhere (B. J. T. et al., unpublished data). Probe S was derived from *fru* coordinates 1–261 plus an additional approximately 340 nt that extend the probe towards the 5′ end of the transcript; probe C was from *fru* coordinates 2783–3607. RNA probes were made from these fragments inserted into Bluescript pSK(+) (Stratagene) and using T7 or T3 polymerase and digoxigenin-labeled UTP according to the DIG RNA labeling kit (Boerhinger-Mannheim). Hybridizations to tissue sections and visualization of hybridized transcripts were carried out as described elsewhere (B. J. T. et al., unpublished data). The numbers of *fru*-positive neurons were determined by counting labeled neurons in complete serially sectioned animals (n = 3 for males and females).

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