Aberrant Splicing and Altered Spatial Expression Patterns in *fruitless* Mutants of *Drosophila melanogaster*

Stephen F. Goodwin,*,§ Barbara J. Taylor,† Adriana Villella,* Margit Foss,† Lisa C. Ryner,‡ Bruce S. Baker‡ and Jeffrey C. Hall*

**Department of Biology, Brandeis University, Waltham, Massachusetts 02454, †Department of Zoology, Oregon State University, Corvallis, Oregon 97331, ‡Department of Biological Sciences, Stanford University, Stanford, California 94305 and §Division of Molecular Genetics, University of Glasgow, Glasgow G11 6NU, United Kingdom*

Manuscript received May 18, 1999
Accepted for publication October 14, 1999

ABSTRACT

The *fruitless* (*fru*) gene functions in Drosophila males to establish the potential for male sexual behaviors. *fru* encodes a complex set of sex-specific and sex-nonspecific mRNAs through the use of multiple promoters and alternative pre-mRNA processing. The male-specific transcripts produced from the distal (P1) *fru* promoter are believed to be responsible for its role in specifying sexual behavior and are only expressed in a small fraction of central nervous system (CNS) cells. To understand the molecular etiology of *fruitless* mutant phenotypes, we compared wild-type and mutant transcription patterns. These experiments revealed that the fru^2, fru^3, fru^4, and fru^{sat} mutations, which are due to *P*-element inserts, alter the pattern of sex-specific and sex-nonspecific *fru* RNAs. These changes arise in part from the *P*-element insertions containing splice acceptor sites that create alternative processing pathways. *In situ* hybridization revealed no alterations in the locations of cells expressing the P1-*fru*-promoter-derived transcripts in fru^2, fru^3, fru^4, and fru^{sat} pharate adults. For the fru^1 mutant (which is due to an inversion breakpoint near the P1 promoter), Northern analyses revealed no significant changes in *fru* transcript patterns. However, *in situ* hybridization revealed anomalies in the level and distribution of P1-derived transcripts: in fru^1 males, fewer P1-expressing neurons are found in regions of the dorsal lateral protocerebrum and abdominal ganglion compared to wild-type males. In other regions of the CNS, expression of these transcripts appears normal in fru^1 males. The loss of *fruitless* expression in these regions likely accounts for the striking courtship abnormalities exhibited by fru^1 males. Thus, we suggest that the mutant phenotypes in fru^2, fru^3, fru^4, and fru^{sat} animals are due to a failure to appropriately splice P1 transcripts, whereas the mutant phenotype of fru^1 animals is due to the reduction or absence of P1 transcripts within specific regions of the CNS.

THE *fruitless* (*fru*) gene of Drosophila functions at the head of a recently identified branch of the sex-determination hierarchy (Ryner *et al.* 1996) that is required for most, if not all, steps of male courtship behavior (for reviews see Hall 1994; Taylor *et al.* 1994; Cobb and Ferveur 1996; Yamamoto *et al.* 1998). In addition to its male-specific function in the sex hierarchy, *fru* also encodes a sex-nonspecific vital function (Ryner *et al.* 1996).

The *fru* locus spans at least 140 kb and produces a complex array of transcripts due to the use of four promoters and alternative splicing (Figure 1, A and B; Ito *et al.* 1996; Ryner *et al.* 1996; L. C. Ryner, unpublished results). Transcripts from the distal promoter (P1) are alternatively spliced near their 5′ termini to generate sex-specific transcripts (Ryner *et al.* 1996; Heinrichs *et al.* 1998). It is thought that the male-specific P1 transcripts encode *fru*'s sex determination function, whereas the transcripts produced from the more proximal promoters encode *fru*'s vital function(s). Alternative splicing at the extreme 3′ end of the *fru* transcripts leads to the inclusion of one of three mutually exclusive exons that encode alternative pairs of zinc fingers (Ryner *et al.* 1996). Fifteen transcript classes are possible if the five identified 5′ ends (two are produced by sex-specific splicing from P1) are combined with all the identified 3′ ends; cDNAs corresponding to seven of these classes have been identified (Ryner *et al.* 1996). However, the potential transcript diversity may be more extensive, due to the discovery of additional *fru* transcripts containing one of several micro-exons (L. C. Ryner, unpublished results). The transcripts from all *fru* promoters have open reading frames that encode proteins related to the BTB-ZF protein family (*cf.* Hu *et al.* 1995) or Ttk subgroup (*cf.* Albagli *et al.* 1995); other members of this family function as sequence-specific transcription factors (Read and Manley 1992; Read *et al.* 1992; von Kalm *et al.* 1994).

Phenotypic analyses of *fruitless* mutants have extended the previously known roles of *fru* in male courtship

Corresponding author: Stephen F. Goodwin, Division of Molecular Genetics, Anderson College, University of Glasgow, Glasgow G11 6NU, United Kingdom. E-mail: stephen@molgen.gla.ac.uk

behavior (Hall 1994; Ryner *et al.* 1996; Villella *et al.* 1997). The courtship actions of wild-type males consist of a series of behaviors: tapping, orientation, following, wing extension, courtship-song production, licking, attempted copulation, and mating (Hall 1994; Cobb and Ferveur 1996; see Table 1 for details). Because this sequence of behaviors is a dependent series of actions, and the most severely affected *fru*-mutant types display virtually no courtship, the question arises whether *fruitless* may only be necessary for an initial step in the courtship sequence. Evidence that the latter interpretation is incorrect, and that instead *fru* is essential for a number of individual steps in courtship behavior, comes from the phenotypes of less severely affected *fru* genotypes. These mutants exhibit defects in certain steps of courtship, but they are able to carry out subsequent steps. For example, fru^1, fru^2, fru^3, and fru^4 homozygotes exhibit defects very early in courtship, in that they cannot discriminate between females and males as appropriate courtship partners, yet they are able to carry out some later steps of courtship, which they direct at both sexes (Gailey and Hall 1989; Gailey *et al.* 1991; Ryner *et al.* 1996; Villella *et al.* 1997), whereas fru^{sat} homozygotes have been reported to exhibit exclusively intermale courtship (Ito *et al.* 1996; Yamamoto *et al.* 1996, 1997, 1998). During a subsequent courtship step, fru^1 and fru^2 males produce a courtship song, but it is abnormal (Wheeler *et al.* 1989; Villella *et al.* 1997), while fru^3 and fru^4 males exhibit some wing extensions but fail to generate song pulses during these wing extensions (Ryner *et al.* 1996; Villella *et al.* 1997). These mutants also differ in their ability to carry out the final steps of courtship in that fru^2 males are fertile, whereas fru^1, fru^3, fru^4, and fru^{sat} males do not attempt copulation. These observations on the phenotypes of various *fru* mutants implicate *fru* in several specific parts of the courtship sequence and not simply the initiation of male sexual behavior.

fruitless exerts its effects on male sexual behavior through its expression in a limited portion of the central nervous system (CNS; Ryner *et al.* 1996). Transcripts from the distal (P1) *fru* promoter are restricted to a few hundred neurons of the adult CNS. Behavioral analyses of gynandromorphs implicated several regions of the CNS in particular steps of male courtship (Hall 1977, 1979; Schilcher and Hall 1979), and our initial assessment of cells expressing the P1 *fru* transcripts (Ryner *et al.* 1996) led to labeling of largely the same neuronal tissues identified in the earlier studies. However, not all regions of the CNS in which the P1 *fru* promoter is expressed have yet been specifically linked to male courtship behavior. Another *fruitless*-derived probe, designed to detect *fru* transcripts produced from all the promoters, showed that most or all neurons in the CNS, as well as many cells in non-neuronal tissues, express this gene (Ryner *et al.* 1996). This more general expression pattern, stemming from the use of one or more of the proximal *fru* promoters, is likely responsible for *fru*'s vital function (Ryner *et al.* 1996).

Here, we focus on the molecular etiology of the phenotypes produced by *fru* alleles that affect male sexual behavior, but not *fruitless*'s vital function. These include four *P*-element mutations (fru^2, fru^3, fru^4, and fru^{sat}), which are inserted between the P1 promoter and the common coding region, and the fru^1 mutation, which is due to an inversion breakpoint just distal to the P1 promoter (Figure 1A). Northern analysis shows that the four *P*-element mutations alter the size of the *fru* transcripts produced from the promoters located distal to the inserts. These changes arise in part from aberrant splicing into acceptor sites present within the *P*-element inserts. Not all transcripts produced from the distal promoters are abnormal in these mutants: reverse transcription (RT)-PCR experiments revealed low levels of RNA species spliced in a normal manner. The finding of some wild-type transcripts in the *P*-element mutations can account for the fact that they differ in the severity of their phenotypes. *In situ* hybridization to tissue sections revealed that the spatial pattern of expression in the *P*-element *fru* mutants was similar to the wild-type male pattern. In contrast, males homozygous for fru^1 make a normal set of sex-specific transcripts, but exhibit an altered spatial pattern of *fruitless* expression: a subset of the cells that express *fru*'s sex-specific transcripts in wild type do not express these transcripts in fru^1 homozygotes. These data strongly support the suggestion (Ryner *et al.* 1996) that neurons expressing *fru*'s sex-specific transcripts in the CNS control male sexual behavior.

MATERIALS AND METHODS

Drosophila strains and culture conditions: Cultures of *Drosophila melanogaster* were reared in 12:12-hr light:dark cycles at 25° and 70% relative humidity on a sucrose-cornmeal-yeast medium containing the mold inhibitor Tegosept. Bottles were cleared of parents after 5 days. Males were collected under mild ether anesthetization 0–6 hr after eclosion and then aged individually on unyeasted autoclaved food (see below). Wild-type Canton-S virgin females were collected 0–6 hr after eclosion, grouped in food vials, and then aged for 1–5 days.

***fruitless* stocks:** fru^1 was balanced with *In(3LR)TM3, Sb (TM3)*. fru^2 contains a w^+, ry^+-marked *P* element, *P{(w^+, ry^+)A}* (Hazelrigg *et al.* 1984), inserted at the *fruitless* locus (Gailey and Hall 1989; Gailey *et al.* 1991); this strain had been outcrossed to a "Cantonized" *white* stock for 11 generations (reextracting w^+ each time) and was subsequently maintained as a homozygous stock (Villella *et al.* 1997). fru^3 and fru^4 arose by insertions of *P{lacZ, ry^+}*, also known as *P{PZ}* within *fru* (Castrillon *et al.* 1993). Both mutations were outcrossed to a ry^{506} stock for four generations, with reextraction of ry^+, then maintained in *MKRS*-balanced stocks (Villella *et al.* 1997). fru^{sat} is caused by a *P{lwB}* insert at the locus (Ito *et al.* 1996; Yamamoto *et al.* 1996, 1997) and was maintained with *In(3LR)TM6B, Hu e.* The three deletions $Df(3R)Cha^{M5}$, *Df(3R)P14*, and $Df(3R)fru^{w24}$ — referred to as Cha^{M5}, *P14*, and fru^{w24}, respectively (Gailey and Hall 1989; Ryner *et al.* 1996) — were used for behavioral observations.

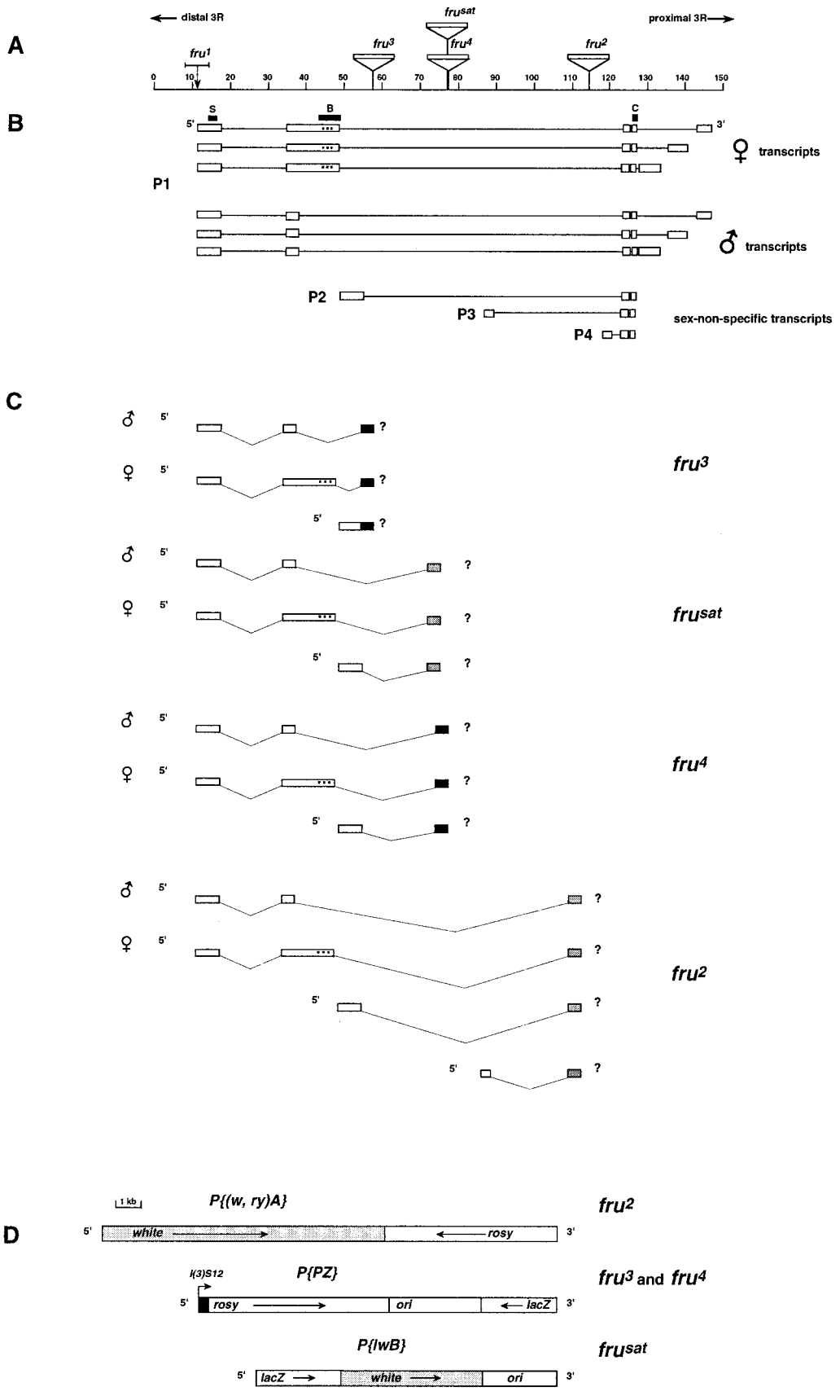

Figure 1.—Genomic map of the *fruitless* gene. (A) The *fru*^{2}, *fru*^{3}, *fru*^{4}, and *fru*^{sat} *P*-element insertion sites are indicated by inverted triangles; the position of the *fru*^{1} breakpoint is indicated by an arrow. The locus spans ~140 kb. (B) Organization of the *fru* transcription units. Open boxes represent genomic fragments that hybridize to *fru* cDNAs; each box may represent more than one exon. The locations of the four promoters (P1–P4) are based on Ryner *et al.* (1996) and L. C. Ryner (unpublished results). Transcripts from the P1 promoter are alternatively spliced near their 5′ termini to generate sex-specific transcript classes. Additional alternative splicing at the 3′ end of the gene inserts one of three mutually exclusive exons (L. C. Ryner, S. F. Goodwin, M. Foss, B. J. Taylor, J. C. Hall, and B. S. Baker, unpublished results). Whether all alternative 3′ ends are used with P2-, P3-, and P4-derived transcripts is unknown. The regions used to generate probes for Northern analyses are indicated by black boxes above the cDNA structures (see materials and methods): S, *5′ sex-specific probe* generated by PCR; B, a 645-bp *Eco*RI genomic fragment containing a cluster of three copies of the 13-nt TRA/TRA-2 repeats contained within this gene; C, BTB domain probe. (C) Structures of aberrant sex and non-sex-specific transcripts produced by *fru*^{2}, *fru*^{3}, *fru*^{4}, and *fru*^{sat} mutants; question marks represent the uncertainty of the cDNAs' 3′ ends. (D) The structure of the transposons *P{(w*$^{+}$*,ry*$^{+}$*)A}*, *P{lwB}*, and *P{lacZ, ry*$^{+}$*}*, also known as *P{PZ}* (Hazelrigg *et al.*, 1984; Wilson *et al.*, 1989; Mlodzik and Hiromi 1992). The directions of transcription are indicated by arrows, the shaded box represents genomic *white* sequences (see text), and the solid box represents genomic *l(3)S12* sequences.

Courtship observations and recordings: For single-pair observations, individual *fru*^{sat} males (7–10 days old) were placed in a recording chamber (*cf.* Villella and Hall 1996; Villella *et al.* 1997) with either another male of the same genotype or a wild-type female (1–5 days old); the fly pairs were video- and audio-recorded for 5–10 min. A courtship index (CI) was calculated for each *fru*^{sat} male as described previously (Villella and Hall 1996; Villella *et al.* 1997). The CI represents the percentage of time the male exhibited courtship behavior, including all readily observable courtship behaviors: orientation, following, wing extension, and attempted copulation (*e.g.*, Villella *et al.* 1997). For male-pair recordings, a CI was determined only for the male that initiated courtship first for at least 20 sec (Villella *et al.* 1997). A wing-extension index (WEI; Villella *et al.* 1997) was also calculated for each male and represents the fraction of time either wing was held out between 45° and 90° to the body (in wild-type courtship, these wing extensions almost always lead to courtship song). Sounds produced by courting males were analyzed as described previously (*cf.* Villella *et al.* 1997; see the legend to Table 1). Separate observations, involving certain features of the courtship sequence that are difficult to discern with the naked eye, were performed at ×20 (see the legend to Table 1). For this, the flies were placed within the food-containing chambers of a plastic "courtship wheel" (*cf.* Villella *et al.* 1997); an individual homozygous *fru*^{sat} male (5–7 days posteclosion) was placed with a male of the same genotype and age or with a wild-type virgin female (1–5 days old). The pairs were observed during 1- to 2-hr periods for up to 3 successive days (<3 days for test males that performed all three behaviors indicated in the legend to Table 1 on the first or second day).

For male chaining observations, males were collected at eclosion, aged individually for 6–7 days, and then eight males were grouped per food vial (Villella *et al.* 1997). A chaining index (ChI) was determined for each group: the ChI is the percentage of time three or more males courted one another in groups during a 10-min observation period (*cf.* Villella *et al.* 1997). The ChI was determined on either day 3 or 4 after grouping, because some of the *fru* male types exhibit more chaining after being together for a few days (Villella *et al.* 1997).

To test male fertility, males were collected at eclosion, grouped (<10 males per food vial), and aged for 5–8 days. Individual males were placed in a food vial with three to five wild-type virgin females. The presence or absence of progeny was scored 7–10 days later. Vials with no progeny and in which the male was dead at the time of scoring were excluded.

Molecular biology: RNA isolation, purification, and Northern analyses were as described previously (Goodwin *et al.* 1997). *fruitless* DNA probes (Figure 1B) were as follows: common-coding region probe (C, which detects all RNAs that have BTB-domain-encoding sequences in common) contains nucleotides from coordinates 2075 to 2422 (with respect to GenBank accession no. U72492; Ryner *et al.* 1996); a distally derived 5′ sex-specific exon probe (S), containing nucleotides (nt) from coordinates 188 to 469 (GenBank accession no. U72492); and *tra*/*tra-2*-repeat probe (B), a 645-bp *Eco*RI genomic fragment of the *fruitless* gene containing a cluster of three copies of a 13-nt repeat (GenBank accession no. U72491; Ryner *et al.* 1996; as described in this citation, these kinds of repeats to which TRA/TRA-2 bind were originally identified in *dsx* transcripts). Other probes were to *white* gene sequences [an 11.7-kb *Eco*RI fragment extending from map positions −5.1 to +6.7 relative to the transcription-start site of the *white* gene, numbered as in Hazelrigg *et al.* (1984) and isolated from the plasmid p*P[(w*$^{+}$*, ry*$^{+}$*)A]*], and to *rosy* (*ry*) sequences [a 7.2-kb *Hin*dIII genomic fragment isolated from the plasmid p*PZ* that contains nucleotides from coordinates 6946–14227 (Mlodzik and Hiromi 1992); the *ry* genomic sequence is under GenBank accession no. Y00308].

RT-PCR: These reactions were performed as described previously (Ryner *et al.* 1996), using either 5 μg of total adult head RNA or poly(A)$^{+}$ RNA and random primers. A total of 10% of the first-strand synthesis was used in a 50-μl PCR, consisting of 50 mm KCl, 10 mm Tris-HCl, pH 8.3, 1.5 mm $MgCl_2$, 0.01% Triton X-100, 200 μm dATP, 200 μm dCTP, 200 μm dGTP, 200 μm dTTP, 0.2 μm of each primer, and 2 units of *Taq* polymerase (Promega, Madison, WI). PCR parameters were 3 min at 94°, then 30 cycles of 1 min at 94°, 1 min at 60°, 3 min at 72°, followed by 5 min at 60° and 20 min at 72°. Secondary nested PCRs were as above and contained 2 μl of the primary PCR product as template. The following gene-specific primers were used: *fruitless* forward primers: *fru-1-for* (5′ GAATTCGAGGACGTGTGACGAT 3′); *P1-for-1* (5′ TCG CATTACGCGGCCTTGGACT 3′); *P2-for-1* (5′ TGCTGCAA AAGAACTCAGTCCGC 3′); *P3-for-1* (5′ TTCGGCACGAG GAACAATCG 3′); *P4-for-1* (5′ CACACACACACACATATC GAGTTCC 3′); *fruitless* reverse primer: *fru-7A-rev* (5′ GGA AAATCGTCTCGAAGTACGGAC 3′); *white* and *rosy* reverse primers: *w-1-rev* (5′ ATTTGCTGAGCGAAAGCTCCTGG 3′); *w-2-rev* (5′ TCCGTTGATATTCATCACGCCCAC 3′); *ry-1-rev* (5′ CACAAGGGATTTGACAATGCCAGG 3′); *ry-2-rev* (5′ CTACGAGTGGCAAGCAAACTCCAAG 3′); *fru*^{4} insert primer: *fru4-PL* (5′ CAATGCTTTCCCCCTCTCTTTC 3′); *P*-element primer: *PL* (5′ GTGTATACTTCGGTAAGCTTCGG 3′); ribosomal-protein 49 (*rp49*) forward and reverse primer: *rp49-rev* (5′ GTGTATTCCGACCACGTTACA 3′); *rp49-for* (5′ TCCTAC CAGCTTCAAGATGAC 3′). Southern analysis and sequencing of PCR products were as described previously (Ryner *et al.* 1996).

Orientation and position of the *P*-element transposons: *fru*^{3} and *fru*^{4} are *P{PZ}* transposons inserted within the *fru* locus in unknown orientations (Figure 1, A and D; Castrillon *et al.* 1993; Ryner *et al.* 1996). Genomic DNA flanking the *fru*^{3} and *fru*^{4} *PZ*-element inserts was cloned by plasmid rescue (Mlodzik and Hiromi 1992). Additional sequences from the nonrescuable side of *fru*^{3} and *fru*^{4} were obtained by inverse PCR (Tower *et al.* 1993). Restriction analysis, Southern blotting, and DNA sequencing established the orientation of a given insert. *fru*^{2}'s orientation relative to genomic sequences was determined by Moses *et al.* (1989); the *white* gene within the *fru*^{2} transposon is transcribed in the same direction as *fruitless*, and the *rosy* gene is transcribed in the opposite direction. *fru*^{sat} arose as an insertion of the *P{lwB}* element close to the site of *fru*^{4} (Figure 1, A and D; Wilson *et al.* 1989; Ito *et al.* 1996; Yamamoto *et al.* 1996). The exact position of the *fru*^{sat} insertion site was mapped utilizing *fru*-specific primers derived from the mapping of *fru*^{4} (see above) in conjunction with *P*-element-specific primers. Sequencing of the PCR products indicated that the *P{lwB}* element in *fru*^{sat} had inserted 34 bp distally to the *fru*^{4} insertion site. In *fru*^{sat}, the mini-*white* gene contained within the transposon is transcribed in the same direction as *fruitless*.

***In situ* hybridizations to tissue sections:** Flies were cryostat sectioned at 20 μm in the horizontal plane; the sections were transferred to slides, fixed for 1 hr in RNase-free 4% paraformaldehyde, rinsed in RNase-free PBS-Tx (140 mm NaCl, 50 mm phosphate buffer, pH 7.4, and 0.1% Triton X-100), dehydrated through a methanol series (25–100%), and rehydrated with PBS-Tx. Sections were acetylated with acetic anhydride in 0.1 m triethanolamine (pH 8.0) and hybridized overnight at 55° with 15–100 ng of labeled riboprobe in 150 μl hybridization buffer (50% formamide, 5× SSC, 0.01% Tween-20, 0.01% tRNA, and 0.005% RNase-free heparin per slide). The sections were washed in buffer (600 mm NaCl, 1 mm EDTA, pH 8.0, 10 mm Tris, pH 8.0, 50% formamide) at 55° and incubated

at 37° with RNaseT1 and RNaseA. For signal visualization, the sections were blocked for 1 hr at 25° in 5% nonfat powdered milk in PBS-Tx, incubated for 1 hr at 25° with antidigoxygenin Fab fragments coupled to alkaline phosphatase (1:1000; Roche Molecular Biochemicals, Indianapolis, IN) in blocking solution and developed with NBT/BCIP (GIBCO BRL, Grand Island, NY) in NTMT (100 mm NaCl, 50 mm $MgCl_2$, 100 mm Tris, 0.1% Triton X-100, and 0.015% levamisole, pH 9.5). The sections were mounted in 80% glycerol and 1% propyl-gallate.

Standard protocols (Roche Molecular Biochemicals) were used to synthesize riboprobes from *fru* cDNA and DNA templates [subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA) or pGEM-T Easy (Promega)]. Linearized probes were synthesized using T3, T7, or SP6 polymerase with digoxygenin-coupled nucleotides (DIG RNA Labeling Kit; Roche Molecular Biochemicals). Two riboprobes were made that detect P1-promoter-derived transcripts: probe S1, derived from a genomic clone (coordinates 1–261 with respect to GenBank accession no. U72491), includes sequences from probe S and an extra 340 nt of genomic sequence. Two common coding-region riboprobes were made: probe C1 (coordinates 2783–3607 with respect to GenBank accession no. U72492) and the overlapping probe C (see above). The S and S1 probes gave identical results, as did the C and C1 probes. For the analysis of *in situ* hybridization patterns, the following numbers of animals were examined: Canton-S males (S1 probe, $n = 18$; C1 probe, $n = 10$), females (S1, $n = 10$; C1, $n = 11$); fru^1 males (S1, $n = 4$; C1, $n = 2$), females (S1, $n = 7$, C1, $n = 2$); fru^2 males (S1, $n = 5$; C1, $n = 8$), females (S1, $n = 3$; C1, $n = 3$); fru^3 males (S1, $n = 3$; C1, $n = 14$), females (S1, $n = 3$; C1, $n = 9$); fru^4 males (S1, $n = 4$; C1, $n = 14$), females (S1, $n = 4$; C1, $n = 8$); and fru^{sat} males (S1, $n = 9$; C1, $n = 14$), females (S1, $n = 6$; C1, $n = 4$).

Statistical analyses: For the courtship data (see legend to Table 1), nonparametric Kruskal-Wallis pairwise comparisons (JMP, Statistics and Graphics Guide, version 3; SAS Institute Inc., Cary, NC) were performed to determine whether there were differences among the CIs or ChIs recorded for fru^{sat} males, or between mutant and fru^+-associated CIs. For the *in situ* hybridizations (see legend to Table 5), the cell counts were analyzed by one-way ANOVA (Statgraphics Version 5.0; Manugistics Inc., Rockville, MD) using 95% Tukey honestly significant difference (HSD) for contrast to compare numbers of labeled neurons between mutant and wild-type animals of the same sex for a particular neuronal group.

RESULTS

fru^{sat} behavior: One objective of these studies was to elucidate the molecular basis for courtship defects caused by viable *fru* mutations. Because fru^{sat} has not been as well characterized behaviorally as the other *fru* mutants (Villella *et al.* 1997), we first quantified the effects of fru^{sat} on male sexual behaviors. fru^{sat} males, when paired with either another fru^{sat} male or a wild-type female, exhibited similar low levels of courtship (Table 1). Hemizygous fru^{sat} males showed levels of courtship comparable to those of fru^{sat} homozygotes (Table 1). Thus the extremely low CIs recorded for fru^{sat} males, compared to the values for wild-type or $fru^{sat}/+$ controls (Table 1), are not due to recessive genetic-background effects. Despite this paucity of courtship, sufficient behavior by fru^{sat} males was observed to reveal that the residual courtship was not normal. During wing extensions, fru^{sat} males produced only extremely brief sine-song bouts; no song pulses were generated (Table 1 legend). Detailed inspections of fru^{sat} male behavior revealed that this mutant exhibits tapping and licking, but no attempted copulation (Table 1 legend). fru^{sat} males, when grouped together, displayed the typical *fruitless* behavior of courtship chaining (*cf.* Gailey and Hall 1989; Villella *et al.* 1997); these males displayed numerous wing extensions toward one another when chaining.

fru^{sat} males were completely sterile when heterozygous with the deletions fru^{w24} ($n = 20$) or *P14* ($n = 16$). However, 1 fru^{sat}/Cha^{M5} male out of 39 was fertile. fru^{sat}/fru^1 males were also weakly fertile (14/30 males); this proportion is similar to those observed when fru^3 or fru^4 are heterozygous with fru^1 (~65% in both cases; Villella *et al.* 1997).

Origins of normal *fruitless* transcripts: When a probe common to all known classes of *fru* cDNAs (Figure 1, probe C) was hybridized to Northern blots of poly(A)$^+$ RNA from sexed wild-type adult heads, three female-specific (9.0, 8.0, and 7.4 kb), three male-specific (7.9, 6.4, and 5.4 kb), and one common (4.4 kb) transcripts were detected (Figure 2A; Ryner *et al.* 1996).

Sex-specific *fru* transcripts are generated by the sex-specific usage of alternative 5′ splice sites that are 1590 nt apart in the P1-derived pre-mRNA (Ryner *et al.* 1996; Figure 1B). Ryner *et al.* (1996) suggested that the three male-specific and three female-specific transcripts are generated from P1-derived transcripts by sex-specific splicing at their 5′ ends and by sex-nonspecific alternative splicing to exons containing three alternative zinc-finger pairs at their 3′ ends. This predicts that the three classes of female-specific transcripts would have common sequences at their 5′ ends, as should the three male-specific forms. To test this, we used a *fru* probe from upstream of the female-specific 5′ splice site (Figure 1, probe B) to probe Northern blots of poly(A)$^+$ RNA from sexed adult heads. We detected only the three female-specific (9.0, 8.0, and 7.4 kb) transcripts (Figure 2B) seen previously; no signals were observed in male head RNA, even with long autoradiographic exposures. These results are consistent with the interpretation that all three female-specific *fru* transcripts share common 5′ female-specific sequences.

The sequences of the sex-specific *fru* transcripts revealed that there are no sequences unique to the male transcripts: both male- and female-specific *fru* transcripts share common sequences from upstream of the male-specific 5′ splice site (Ryner *et al.* 1996). We therefore used a probe from this region (Figure 1, probe S) on Northern blots of poly(A)$^+$ RNA from sexed wild-type heads (Figure 2C); only the three female sex-specific transcripts (9.0, 8.0, and 7.4 kb) and three male sex-specific transcripts (7.9, 6.4, and 5.4 kb) were observed. This set of results provides strong support for the conclusion that these six sex-specific *fru* transcripts

TABLE 1

Male courtship behavior affected by the *fru^{sat}* mutation

Genotype	Indices	Behavior: Mutant or control male courting: Another male	Behavior: Mutant or control male courting: A female	Behavior: Group intermale courtship (ChI)
fru^{sat}/fru^{sat}	CI	5 ± 2	3 ± 1	17 ± 2
	WEI	1 ± 1	0 ± 0	(8)
		(15)	(13)	
fru^{sat}/Cha^{M5}	CI	6 ± 2	8 ± 4	27 ± 4
	WEI	0 ± 0	1 ± 1	(10)
		(15)	(12)	
fru^{sat}/P14	CI	16 ± 6	6 ± 4	7 ± 3
	WEI	4 ± 3	2 ± 1	(9)
		(14)	(12)	
fru^{sat}/fru^{w24}	CI	8 ± 5	1 ± 1	36 ± 7
	WEI	1 ± 1	0 ± 0	(10)
		(12)	(12)	
fru^{+} (wild type)	CI	3 ± 1	74 ± 5	0 ± 0
	WEI	0 ± 0	37 ± 5	(5)
		(10)	(10)	
fru^{sat}/+	CI	2 ± 1	83 ± 2	0 ± 0
	WEI	0 ± 0	46 ± 3	(5)
		(12)	(12)	

CIs (courtship indices) ± SEM, mean percentage of time that the test males spent courting another male or a female in 5- to 10-min observation periods (see materials and methods); WEIs (wing-extension indices), mean percentage of time the males displayed wing extensions toward the other male or a female (wild type, Canton-S) during such an observation period. For male-male courtships, only the first fly to initiate courtship had his actions quantified (the other male in an observation of this kind was of the same genotype as the recorded courter). The numbers of fly pairs observed are in parentheses (first two data columns). ChI (chaining index, last data column), mean percentage (± SEM) of time at least three males of the eight (per tested group) showed courtship toward each other in a 10-min observation period; the numbers of the eight-male groups observed are in parentheses. The *fru^{+}* control values are from observations of Canton-S males and the *fru^{sat}/+* control males resulting from a cross of mutation-bearing to Canton-S flies. Statistical comparisons (see materials and methods) revealed that *fru^{sat}* males courted others at the same levels as did *fru^{sat}/P14*, *fru^{sat}/Cha^{M5}*, and *fru^{sat}/fru^{w24}* ($P \geq 0.05$ in all cases); the same kind of comparison with respect to male-with-female behavior—between the effects of *fru^{sat}* homozygosity and uncoverage of its effects by the *Df*s—revealed no differences ($P \geq 0.05$ in all cases). Comparing the behavior of *fru^{sat}/fru^{sat}* and *fru^{sat}/Df* types (together) in terms of courting other males *vs.* females, there were no differences ($P \geq 0.05$ in all cases). In the male-with-male tests, these mutant types courted other males at the same levels as did wild-type or *fru^{sat}/+* males ($P \geq 0.05$ in all cases). During a total of *ca.* 9 min, during which time *fru^{sat}/fru^{sat}* and *fru^{sat}/Df* males extended their wings toward females or males (given the WEI values in the first two data columns), no song pulses were recorded, whereas *fru^{+}*-bearing males generated 200–400 pulses per minute (Villella *et al.* 1997). From the current recordings of male-male pairs, only four homozygous *fru^{sat}* males displayed wing extensions toward another male (out of 15 mutant individuals tested). Two of these four males generated only brief low-amplitude sine-song bouts; such humming sounds were within the normal frequency range (*cf.* Villella *et al.*, 1997). Although homozygous *fru^{sat}* males showed little interest in wild-type females, in 2 of the 13 cases, the male followed, oriented, and displayed wing extensions toward the female, but produced no sounds. Statistical comparisons of ChIs revealed that homozygous *fru^{sat}* males chained as much as *fru^{sat}/Cha^{M5}* and *fru^{sat}/fru^{w24}* did ($P = 0.09$ and 0.07, respectively); *fru^{sat}/P14* males chained significantly less than homozygous *fru^{sat}* males ($P = 0.02$). Additional observations, made at high magnification to determine whether steps in the courtship sequence occurred in addition to those tabulated, showed that 7 of 20 *fru^{sat}/fru^{sat}* males tested with females initiated courtship by tapping the latter with their foreleg. Two of these seven executed a late step in the sequence by attempting to lick her abdomen with proboscis extension; one mutant male licked with genital contact. None of the 20 *fru^{sat}* males attempted copulation. All wild-type males ($n = 10$) tested with females tapped, licked (7 of 10 with contact), and attempted copulation. The high-magnification observations of *fru^{sat}* male pairs ($n = 15$) showed that 14 of the test individuals (the "courters," defined as above) exhibited tapping behavior; 7 of 14 proceeded to licking (1 of 7 with contact), and no attempted copulation was observed.

arise from sex-specific splicing at the 5′ end of transcripts produced from the distal (P1) *fru* promoter (Figure 1).

Molecular etiology of *fru* mutant phenotypes: Because the viable *fru* alleles are due to lesions outside of coding sequences (Figure 1), we suspected that they might have effects that would be detectable at the transcript level. Northern blots hybridized with the common coding region probe (Figure 1, probe C) showed that in the case of the *fru^{1}* mutant, the relative levels of the male- and female-specific transcripts were not appreciably different from those of wild-type controls (Figure 3). Northern blots involving RNA from the four *P*-element mutants, hybridized with the common coding region probe (C), revealed that sex-specific differences in the pattern of *fru* expression were no longer detected (Fig-

ure 3). Instead, transcripts of ~7.4 and 4.4 kb were detected in both males and females (Figure 3, A–C). In addition to the anomalous 7.4-kb transcript detected in all four mutants, a novel 3.0-kb transcript was observed in *fru*^{3}, *fru*^{4}, and *fru*^{sat}. These results suggest that, in the four *P*-element mutants, sequences from the distal P1 promoter are no longer sex-specifically spliced to the common-coding region.

To characterize further the effects of these mutations, we rehybridized the Northern blots shown in Figure 3 (A–C) with a probe from the 5′ end of P1-derived transcripts (Figure 1, probe S). Again, in the *fru*^{1} mutant, the *fru* transcript pattern appears to be normal (Figure 3D). In the four *P*-element mutants, two patterns of transcripts were seen. First, in *fru*^{2} and *fru*^{sat}, there is an abundant 4.0-kb transcript in males and an abundant 6.0-kb transcript in females (Figure 3, E and F; longer exposures revealed a faintly hybridizing band at 1.35 kb in *fru*^{sat}); none of these corresponds to a transcript size seen in wild type. Second, *fru*^{3} and *fru*^{4} also produce apparently identical arrays of transcripts; these include two major RNA species, a male-specific 2.0- and a 4.0-kb transcript, found in both sexes (Figure 3D). In addition, there are three minor bands common to *fru*^{3} and *fru*^{4} males and two different minor bands common to *fru*^{3} and *fru*^{4} females. Taken together, the above findings indicate that the array of transcripts in *fru*^{1} males and females is normal, but a novel array of P1-derived transcripts is produced in all the *P*-element mutants.

The finding of a transcript apparently common to the two sexes in *fru*^{3} and *fru*^{4} is surprising, because all other transcripts detected in these mutants, as well as wild-type with this probe, are sex specific. We therefore wondered whether the 4.0-kb transcripts seen in *fru*^{3} and *fru*^{4} males and females are really identical, or whether they were only fortuitously of the same size. We therefore reanalyzed the Northern shown in Figure 3 with a *fru* probe from upstream of the female-specific 5′ splice site (Figure 1, probe B), which in the wild type detected only the three female-specific transcripts (Figure 2B). In the *fru*^{3} and *fru*^{4} mutants, this probe hybridized only to a single female-specific transcript of 4.0 kb; no transcripts were detected in males. These results indicate that the 4.0-kb transcripts observed in *fru*^{3} and *fru*^{4} females and males are two molecularly distinct species.

There are several striking features of these Northern results:

1. *P* elements inserted at different locations within a 60-kb region of the large *fru* intron all lead to the production of novel *fruitless* transcripts.
2. The *fru*^{2} and *fru*^{sat} mutants produce identical arrays of novel *fru* transcripts despite the fact that these insertions are *ca.* 40 kb apart (Figure 1).
3. Similarly, the *fru*^{3} and *fru*^{4} mutations also generate identical arrays of novel *fru* transcripts. A possible basis for the different effects of these two pairs of *fru* mutants is that *fru*^{3} and *fru*^{4} are due to *P*-element

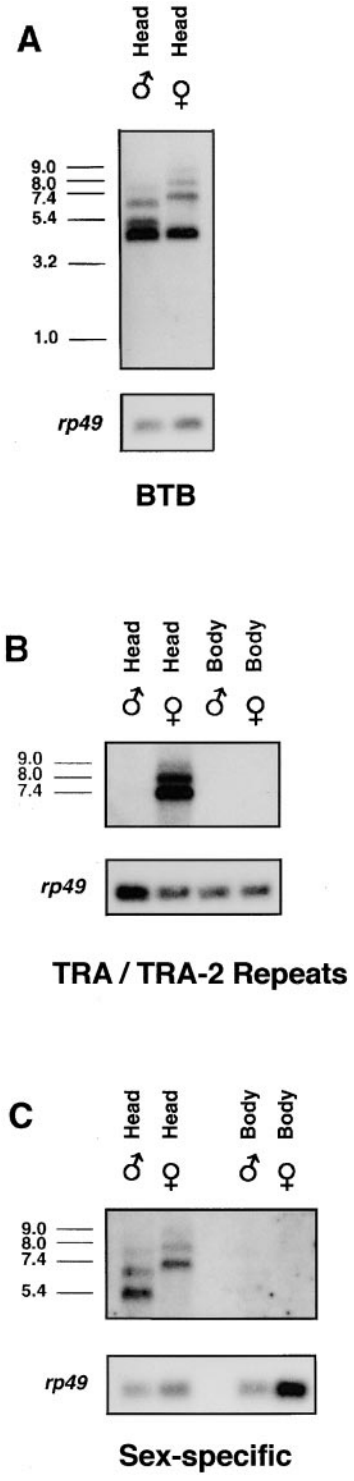

Figure 2.—Northern blot analysis of *fruitless* mRNA from sexed head and body tissues of wild-type flies. A total of 5 μg of poly(A)$^{+}$ RNA was separated in each lane. Hybridization was performed with the following probes (see Figure 1B): (A–C) probes C, B, and S, respectively. Sizes of the transcripts in kilobases are on the left. These blots were subsequently probed with *rp49* (O'Connell and Rosbash 1984) to control for loading differences among lanes (see bottom of figure).

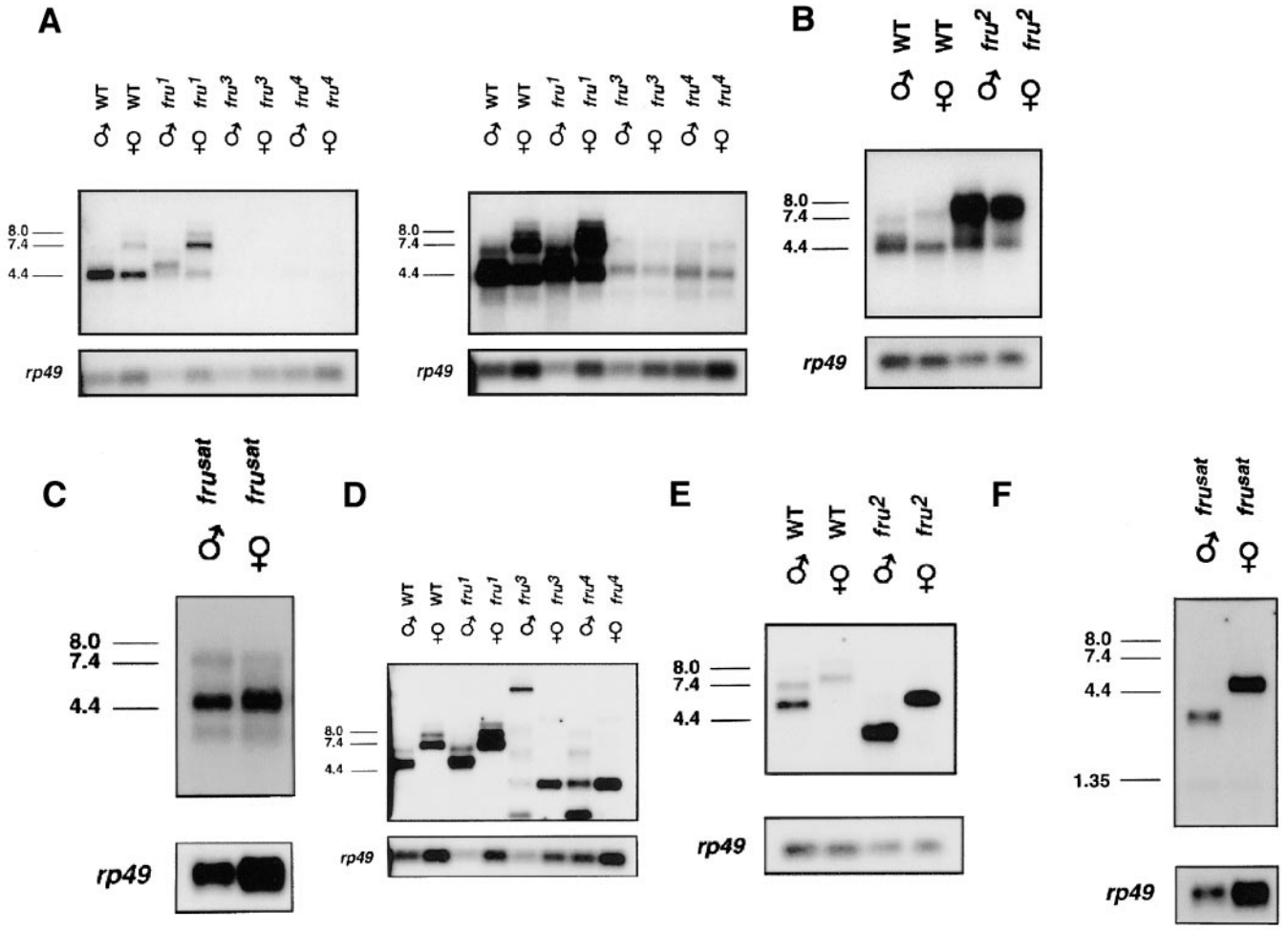

Figure 3.—Northern blot analysis of *fruitless* sexed head mRNA from wild-type (WT) and *fruitless* mutants. A total of 5 μg of poly(A)$^+$ RNA was separated in each lane. Hybridizations were performed with the following probes (see Figure 1B): (A–C) probe C (right part of A is a longer exposure), (D–F) probe S; on the left, the transcript sizes are indicated in kilobases. These blots were subsequently probed with *rp49* to assess loading. In D, the one high-molecular-weight transcript in *fru*^{3} males is of unknown origin; it was not detected on a subsequent blot of RNA derived from an independent RNA sample (data not shown).

insertions of the same transposon, *P{PZ}*, while *fru*^{2} and *fru*^{sat} are due to the insertion of different *P*-element transposons [*P{(w*$^+$*, ry*$^+$*)A}* and *P{lwB}*, respectively] that share some sequences in common.

RT-PCR analysis of the transcripts generated in *P*-element *fru* mutants: We used RT-PCR to investigate whether the four transposon inserts lead to splicing between *fru* sequences transcribed from a given *fru* promoter and sequences in the respective downstream transposon. Because potential splice-acceptor sites within the inserted elements depend on the orientation of the transposon relative to *fru*, we first determined the orientations of the *fru*^{3} and *fru*^{4} inserts (see materials and methods). The *PZ* elements in *fru*^{3} and *fru*^{4} are oriented such that the *rosy*$^+$ marker gene is expressed from the same strand as *fru*. In the case of *fru*^{2} and *fru*^{sat}, the *P* element inserted in these mutants carries part of the *white* gene, including its first intron and the splice sites that flank it. We confirmed previous reports that these two elements are inserted such that the sense strands of *fru* and the *white* gene in the *P* elements are the same (*cf.* Moses *et al.* 1989; Ito *et al.* 1996).

The positions of the *fru* promoters relative to the *P*-element insertions (Figure 1A) suggest which transcripts might be affected in each mutant. For example, sequences from *fru*^{sat}, which is located between P2 and P3 (Figure 1C), would be expected to be included in transcripts originating from the P1 and P2, but not the P3 and P4 promoters, and, thus, might alter the processing of P1- and P2-derived transcripts. Because the Northern results led us to expect the effects of *fru*^{2} and *fru*^{sat} to be similar to one another, and different from those of *fru*^{3} and *fru*^{4}, we describe results from these two pairs of mutants separately.

To determine whether there are transcripts being produced in *fru*^{2} and *fru*^{sat} that fuse *fru* sequences with *white* sequences from within these transposons, RNA was isolated from these mutants and subjected to RT-PCR; the primer-set combinations represented each *fru* pro-

moter (P1–P4) as well as nested *white*-gene-specific primers (see materials and methods). PCR products were sequenced directly. The nucleotide sequences of such products showed that *fru* P1 transcript sequences are spliced to those of *white* in both mutants in each sex (Figure 4A). These splices utilize the normal fru^+ male- and female-specific 5′ splice donor sites, which are spliced to a common acceptor site >70 kb downstream in wild-type P1 transcripts. However, in fru^2 and fru^{sat} mutants, these 5′ splice sites are joined to the normal acceptor site in the first intron of the *white* gene of the *P{(w+, ry+)A}* and *P{lwB)* inserts, respectively (Pepling and Mount 1990). Conceptual translation of these mRNAs showed that FRU amino acids are fused out of frame with *white* sequences (Figure 4A).

We also detected aberrant transcripts derived from the other *fru* promoters located distal to these inserts. In fru^2 mutants, transcripts from P2 and P3 promoters are spliced via the normal *fru* 5′ splice donor sites (L. C. Ryner and S. F. Goodwin, unpublished results) to the normal acceptor site in the first intron of the *white* gene of the *P{(w+, ry+)A}* (Figure 4, B and C), whereas P4 transcripts are normal. In fru^{sat} mutants, as predicted from its enhancer trap location between P1 and P2, P2 transcripts are spliced to *white* sequences from the *P{lwB}* insert (Figure 4B), whereas P3 and P4 transcripts appear to be normal.

Both the fru^3 and fru^4 inserts are located between the P2 and P3 promoters and, thus, might be expected to affect transcripts from the P1 and P2 promoters. Horowitz and Berg (1995) previously showed that the *Hin*dIII fragment of the *rosy* gene that was used to make the *PZ* element (Mlodzik and Hiromi 1992) contains a portion of another gene, *l(3)S12* (located adjacent to *rosy*), including a portion of its intron, the splice-acceptor site, and some coding and transcription-termination sequences. To ascertain whether aberrant transcripts are produced in fru^3 and fru^4 as a result of splicing of *fru* transcripts into the *rosy* or *l(3)S12* genes, we employed RT-PCR analysis with gene-specific primer sets representing each *fru* promoter (P1–P4), with primers based on the *rosy* and *l(3)S12* gene sequences (see materials and methods). The nucleotide sequences of the PCR products demonstrated that sequences from the *fru* P1 promoter are spliced to those of *l(3)S12* in both mutants (Figure 4D). This splice utilizes the normal fru^+ male- and female-specific 5′ splice donor sites in males and females, respectively, which are joined to a sequence in *l(3)S12* previously identified as a splice-acceptor site (Horowitz and Berg 1995). Conceptual translation of these fusion cDNAs showed that *fru* sequences are fused out of frame with the *l(3)S12* ORF (Figure 4D).

We also examined the effects of the inserts in fru^3 and fru^4 on the processing of transcripts from the other *fru* promoters. Aberrant transcripts derived from the P2 *fru* promoter were detected in both fru^3 and fru^4. Transcripts from the P2 promoter are spliced via the normal *fru* 5′ splice donor site (L. C. Ryner and S. F. Goodwin, unpublished results) to the previously identified 3′ splice site in *l(3)S12* of the *PZ* element present in these two mutants (Figure 4E). In both fru^3 and fru^4, processing of transcripts from the P3 and P4 promoters is normal, as predicted from the locations of these promoters downstream of the insertion sites in these two mutants. Thus, in fru^3 and fru^4, as in fru^2 and fru^{sat}, processing of all transcripts initiating upstream of a *P*-element insertion site is disrupted, whereas processing of transcripts initiated from downstream promoters is normal.

The findings that sequences in all four transposons lead to nonproductive splicing of *fru* transcripts offers a first-order explanation for how transposon inserts generate *fru*-mutant phenotypes. However, what is not accounted for by these findings is that these four insertion mutations differ in the severity of their phenotypic effects. One possible explanation for these phenotypic differences is that there are varying levels of normal *fru* transcripts generated from the P1 promoter in these mutants. Although normal *fru* P1 transcripts were not detected in our Northern analysis (see above), it is possible that such transcripts are present, but at levels below those detectable by Northern analysis (∼1–10 pg of transcript; *cf.* Sagerström and Sive 1996). We therefore used the more sensitive technique of RT-PCR (detection limit is 0.01–0.1 pg of transcript; Sagerström and Sive 1996) to ask whether normal *fruitless* transcripts are generated in these mutants.

RT-PCR experiments utilizing total RNA failed to detect wild-type *fru* transcripts from the P1 or P2 promoters in the *P*-element mutants, in contrast to wild-type controls (data not shown; see materials and methods). In addition, P4 sex-nonspecific transcripts were detectable in all the mutants tested; P3 transcripts were detected in fru^3, fru^4, and fru^{sat} males, but not in fru^2 males. This experiment was repeated twice using different RNA isolates, and the RNA's integrity in the RT-PCR reaction was checked using primers derived from the *rp49* gene (see materials and methods); in all samples, we observed amplification of *rp49* sequences. In a further effort to identify normal *fru* transcripts in these mutants, we used head poly(A)+ mRNA isolated from wild-type male and female heads (separately)—and from sexed heads of fru^2, fru^3, fru^4, and fru^{sat} flies—for RT-PCR analysis. Sequencing of PCR products was used to confirm their identity. Although these RT-PCR experiments were not quantitative, we were able to detect the normal array of spliced *fru* products from all four promoters in all the *P*-element mutants (data not shown).

CNS expression patterns in *fru* mutants: An important feature of P1-derived transcripts is that they are expressed in several hundred neurons in the CNS of adults (Ryner *et al.* 1996). We analyzed the spatial pattern of P1 expression in wild type in more detail and, at this

A

fru*² and *fru*ˢᵃᵗ P1 *white

```
                    20                  40                  60
ATGATGGCGACGTCACAGGATTATTTTGGCAATCCATACGCCCTTTTCCGTGGACCGCCC
 M  M  A  T  S  Q  D  Y  F  G  N  P  Y  A  L  F  R  G  P  P

                    80                 100                 120
ACAACTCTGCGGCCGCGCGAGTCGCCGCTGGGCGTGGGCCACCCTCACGGTCATGGGCAC
 T  T  L  R  P  R  E  S  P  L  G  V  G  H  P  H  G  H  G  H

                   140                 160                 180
CTGCACAGCCACGCCCATGCCCACGGGCACGGCCACGCCCACTCGCATTACGCGGCCTTG
 L  H  S  H  A  H  A  H  G  H  G  H  A  H  S  H  Y  A  A  L

                   200                 220                 240
GACTTGCAGACGCCGCACAAGCGGAACATCGAAACGGATGTGCGGGCACCGCCGCCGCCA
 D  L  Q  T  P  H  K  R  N  I  E  T  D  V  R  A  P  P  P  P

                   260                 280 ↓               300
TTGCCGCCGCCACCGCTTCCGCTTCCGCCCGCATCCCCTAGGGTGACAGCGGAGCGGCTT
 L  P  P  P  P  L  P  L  P  P  A  S  P  R  V  T  A  E  R  L

                   320                 340                 360
CGCAGAGCTGCATTAACCAGGGCTTCGGGCAGGCCAAAAACTACGGCACGCTCCTGCCAC
 R  R  A  A  L  T  R  A  S  G  R  P  K  T  T  A  R  S  C  H

                   380                 400
CCAGTCCGCCGGAGGACTCCGGTTCAGGGAGCGGCCAACTAG
 P  V  R  R  R  R  T  P  V  Q  G  A  A  N  *
```

B

fru*² and *fru*ˢᵃᵗ P2 *white

```
                    20                  40                ↓ 60
AATCGCTCGGTTTTAGTTTCCCATAAATGCTGCAAAAGAACTCAGTCCGCAAGGTGACAG
                                  M  L  Q  K  N  S  V  R  K  V  T

                    80                 100                 120
CGGAGCGGCTTCGCAGAGCTGCATTAACCAGGGCTTCGGGCAGGCCAAAAACTACGGCAC
 A  E  R  L  R  R  A  A  L  T  R  A  S  G  R  P  K  T  T  A

                   140                 160
GCTCCTGCCACCCAGTCCGCCGGAGGACTCCGGTTCAGGGAGCGGCCAACTAG
 R  S  C  H  P  V  R  R  R  T  P  V  Q  G  A  A  N  *
```

C

fru*² P3 *white

```
                    20                  40                  60
TTCGGCACGAGGAACAATCGCAACACATGCGACAGTGCAACATGTGGCGCCTCGCTGCCT

                    80                 100                 120
AAAAGCAAAAAGATAATCAAAGAAAAAGCATTGTGTAATAATTTGCACAAAGGAATTCCA

                   140                 160                 180
AACTGAGAACGTGCGCGAGTGTTTGCTACAAAGTGAGTGAGATACAATCGCAGAAATATC

                   200                 220                 240
ATCAGCAAATGCCTCGTCGCATCAATGTGCCAGTTGAACTTGAGAGATACAGCTGCATAC

                   260                 280                 300
TCCAGATAATCGAATAATCGAAATACCGAAAGATACAGATACTTTGCCCCGAACCACAAA

                   320                 340                 ↓
CGCAGTGCAAATGTTATAGTGCTTTACCAACTACCTTCAATTGCGGAAATGACAAGGGTG
                                                  M  T  R  V

                   380                 400                 420
ACAGCGGAGCGGCTTCGCAGAGCTGCATTAACCAGGGCTTCGGGCAGGCCAAAAACTACG
 T  A  E  R  L  R  R  A  A  L  T  R  A  S  G  R  P  K  T  T

                   440                 480                 500
GCACGCTCCTGCCACCCAGTCCGCCGGAGGACTCCGGTTCAGGGAGCGGCCAACTAG
 A  R  S  C  H  P  V  R  R  R  R  T  P  V  Q  G  A  A  N  *
```

D

fru*³ and *fru*⁴ P1 *l(3)S12

```
                    20                  40                  60
ATGATGGCGACGTCACAGGATTATTTTGGCAATCCATACGCCCTTTTCCGTGGACCGCCC
 M  M  A  T  S  Q  D  Y  F  G  N  P  Y  A  L  F  R  G  P  P

                    80                 100                 120
ACAACTCTGCGGCCGCGCGAGTCGCCGCTGGGCGTGGGCCACCCTCACGGTCATGGGCAC
 T  T  L  R  P  R  E  S  P  L  G  V  G  H  P  H  G  H  G  H

                   140                 160                 180
CTGCACAGCCACGCCCATGCCCACGGGCACGGCCACGCCCACTCGCATTACGCGGCCTTG
 L  H  S  H  A  H  A  H  G  H  G  H  A  H  S  H  Y  A  A  L

                   200                 220                 240
GACTTGCAGACGCCGCACAAGCGGAACATCGAAACGGATGTGCGGGCACCGCCGCCGCCA
 D  L  Q  T  P  H  K  R  N  I  E  T  D  V  R  A  P  P  P  P

                   260                 280 ↓               300
TTGCCGCCGCCACCGCTTCCGCTTCCGCCCGCATCCCCTAGAGCTTCGTTATCTTCGGAC
 L  P  P  P  P  L  P  L  P  P  A  S  P  R  A  S  L  S  S  D

                   320                 340                 360
GCGATGTGGCGCAGATCCCGTGCTTTCGTAACAGTTTTCTCTACGGAATCAGCGGAGGAA
 A  M  W  R  R  S  R  A  F  V  T  V  F  S  T  E  S  A  E  E

                   380
TCGGCATCGGCTTGCTGA
 S  A  S  A  C  *
```

E

fru*³ and *fru*⁴ P2 *l(3)S12

```
                    20                  40                ↓ 60
AATCGCTCGGTTTTAGTTTCCCATAAATGCTGCAAAAGAACTCAGTCCGCAAAGCTTCGT
                                  M  L  Q  K  N  S  V  R  K  A  S

                    80                 100                 120
TATCTTCGGACGCGATGTGGCGCAGATCCCGTGCTTTCGTAACAGTTTTCTCTACGGAAT
 L  S  S  D  A  M  W  R  R  S  R  A  F  V  T  V  F  S  T  E

                   140
CAGCGGAGGAATCGGCATCGGCTTGCTGA
 S  A  E  E  S  A  S  A  C  *
```

Figure 4.—Nucleotide and predicted amino-acid sequences from the truncated P1–P3 transcripts produced by *fruitless* transposon-insert mutants. *fru* 5′-end sequences are underlined (GenBank accession no. U72492; L. C. Ryner, S. F. Goodwin, M. Foss, T. Carlo, A. Villella, B. J. Taylor, J. C. Hall and B. S. Baker, unpublished results), stop codons are marked with asterisks, and *fru* 5′ splice sites are joined to the normal acceptor site in the first intron of the *white* gene of the *P{(w⁺, ry⁺) A}* and *P{lwB}* inserts, respectively, at positions 80–81 (coordinates with respect to GenBank accession no. X51749; Pepling and Mount 1990). (A) Nucleotide and predicted amino-acid sequences from the hybrid P1 transcripts produced by *fru*² and *fru*ˢᵃᵗ mutant males. The positions of the fusions between *fru* and *white* sequences are indicated by vertical arrows (out-of-frame *white*-encoded amino acids are italicized). (B) Nucleotide and predicted amino-acid sequences from the hybrid P2 transcripts produced by *fru*² and *fru*ˢᵃᵗ males. (C) Nucleotide and predicted amino-acid sequences from the hybrid P3 transcript produced by *fru*² males. (D) Nucleotide and predicted amino-acid sequences from the hybrid P1 transcripts from *fru*³ and *fru*⁴ males; the position of the fusion between the *fru* and *l(3)S12* sequences is indicated by a vertical arrow (out-of-frame *l(3)S12*-encoded amino acids are italicized). (E) Nucleotide and predicted amino-acid sequences for the hybrid P2 transcript from *fru*³ and *fru*⁴ mutants.

higher level of resolution, examined whether any of the viable *fru* alleles alter the gene's spatial expression pattern.

In situ hybridization to P1 transcripts (using the S1 probe; Figure 1) revealed that P1 expression in wild-type pharate adults was largely localized to nine neuronal clusters in the brain and ventral nerve cord (Figure 5, A and B). Labeled neurons were considered part of a group if the signal-containing cells were adjacent to or within a few cell diameters of other similarly sized labeled cells. By these criteria, we identified six neuronal groups, ranging from 10 to 50 neurons per group, which are found in similar locations within male and female brains (Figure 5A), and three neuronal groups in the ventral nerve cord that are male specific (Figure 5B). All nine groups are present as bilateral pairs.

In the brain, the locations of the six groups are as follows: 1, a large group in the dorsal posterior protocerebrum, medial and ventral to the mushroom bodies; 2, a lateral group in the protocerebrum, anterior to the medullary division of the optic lobes; 3–5, three anterior groups in the protocerebrum which are subdivided into lateral (3), intermediate (4), and medial (5) sets; and 6, one anterior group near the mechanosensory part of the antennal lobe (Figure 5A; *cf.* Ryner *et al.* 1996). In the ventral nerve cord, the three groups of male-specific *fru* P1-expressing neurons are as follows: 7, a lateral group between the prothoracic and mesothoracic neuromeres and ventral to the wing neuromere; 8, a ventromedial group in the mesothoracic ganglion; and 9, a ventral group in the abdominal ganglion (Figure 5B). In addition to these groups of cells, both sexes have a small population of labeled neurons that are found as singletons or are too widely separated from other labeled cells to recognize them as belonging to a group (*cf.* Ryner *et al.* 1996).

In contrast to the restricted pattern of expression of P1 *fru* transcripts, a probe (C1) that detects the protein-coding region common to all *fru* transcripts (Figure 1) revealed expression in virtually all neurons in the CNS and in several other tissues (*cf.* Ryner *et al.* 1996). Most cells in the CNS have a relatively low level of expression, whereas a small number of neurons display markedly higher levels of *fru* expression. By their location and number, the subsets of neurons labeled by the C1 probe that have relatively high levels of *fru* expression appear to correspond to the nine groups of neurons detected by the S1 probe (*cf.* Ryner *et al.* 1996; B. J. Taylor, unpublished results).

To determine whether there were changes in the cellular pattern of *fru* expression in the five viable *fruitless* mutants, we carried out analogous *in situ* hybridizations with both the S1 and C1 probes. Only the presence and relative size of the aforementioned nine groups of labeled neurons were analyzed, because these groups of *fru*-expressing cells are distinct and could be unambiguously identified in tissue sections.

Examination of the expression pattern of P1-derived transcripts in fru^1 males revealed distinct groups of labeled neurons in only four of the nine regions where cells expressing these transcripts are found in wild-type males. The four groups of neurons detected were those in the dorsal posterior protocerebrum (1), the optic lobe (2), the antennal lobe (Figure 6, A and B, group 6), and the ventro-medial mesothoracic groups (Figure 5A, group 8). The numbers of labeled neurons in the antennal lobe and dorsal posterior groups in fru^1 males were not significantly different from wild type (Table 2).

The other five groups of cells detected in wild-type males were very difficult to detect in fru^1 males. In the anterior protocerebrum, the normal pattern for three groups of neurons (3–5) was not observed (Figure 6, F and G). In the dorsal anterior protocerebral region, only about one-quarter of the expected number of labeled neurons was detected (Table 2). These labeled neurons were distributed throughout the medial, intermediate, and lateral subdivisions, suggesting that fru^1 leads to a reduced number of cells expressing P1 transcripts in all three regions. It was not possible to identify definitively the remaining labeled neurons as belonging to one of the three anterior protocerebral groups. Likewise, labeled neurons were difficult to detect in two of the male-specific neuronal groups in the ventral nerve cord of fru^1. A small cluster of neurons, $\sim$15% of the expected number, was found in the ventral area, between the prothoracic and mesothoracic neuromeres (Figure 6, K and L; Table 2). By contrast, almost no labeled cells were found in the ventral abdominal region, where group 9 neurons are observed in wild-type males (Figure 6, O and P; Table 2). The reduced numbers of cells expressing P1 transcripts in fru^1 males may be caused by a reduction in transcription from the P1 promoter, instability of these transcripts, or the loss of neurons that normally express these transcripts.

When a probe (C1) common to all *fruitless* transcripts was used, all neurons in fru^1 male CNSs were labeled at a low level, comparable to what is seen in the wild-type CNS. In addition, subsets of neurons showed relatively high levels of *fru* expression. Neurons with such heavy labeling were detected in the dorsal posterior protocerebrum (1), optic lobe (2), antennal lobe (6), and mesothoracic groups (8); these are the same regions in which the neurons expressing the sex-specific transcripts are abundant in fru^1 males (see above). In fru^1, fewer-than-normal numbers of heavily labeled neurons were detected with the C1 probe in the anterior protocerebrum (3–5) as well as within the thoracic (7) and the abdominal ganglion (9) groups (data not shown). These regions are the same as those in which fewer or no labeled neurons were found by *in situ* hybridization with the S1 probe (Figures 5, A and B, and 6, A, B, F, G, K, L, O, and P).

We also used *in situ* hybridization to determine whether the spatial expression of P1-derived transcripts

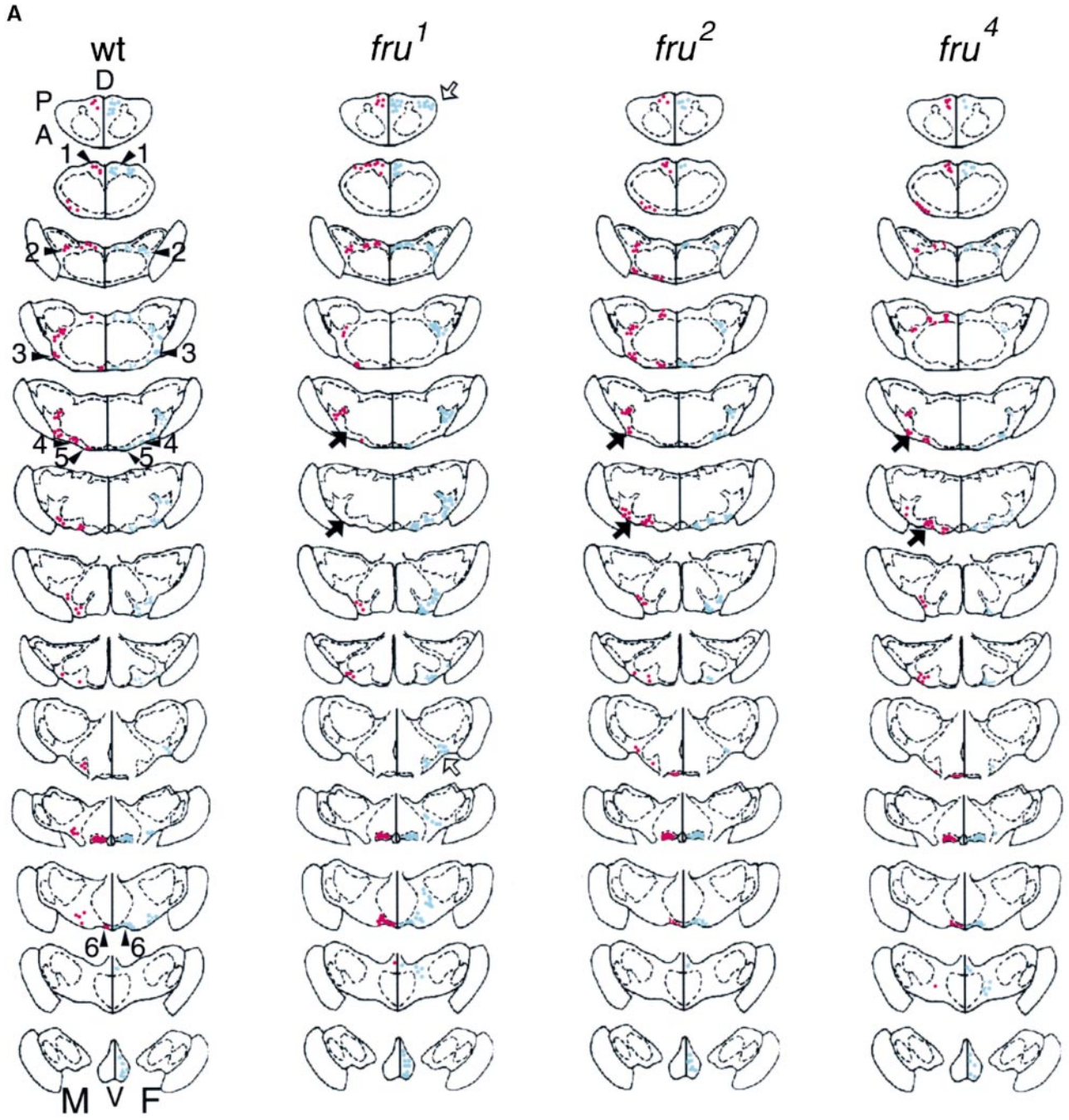

Figure 5.—Distributions of neurons expressing sex-specific *fruitless* transcripts in the nervous system of wild-type and *fru*-mutant males and females. These schematic representations of the CNS are from camera-lucida drawings of a complete series of 20-μm horizontal sections through a wild-type, late-stage pharate adult; the same diagrams were used as templates for expression patterns in the fru^1, fru^2, and fru^4 mutants. The positions of labeled neurons are based on *in situ* hybridizations performed with the S1 probe to serial sections of a single individual of a particular genotype (see Figure 1; materials and methods). The positions of labeled cells were mapped onto the appropriate section, in relation to morphological features visualized with Normarski optics. Neurons in males are indicated by red dots on the left side and in females by blue dots on the right side of the section. The locations of the groups of labeled neurons described in the text are indicated by numbers on the male half of the sections. Because the expression patterns of labeled neurons were similar among fru^3, fru^4, and fru^{sat}, only those sites for a fru^4 brain are shown. In the brain series in Figure 5A, the first section is the most dorsal (D) one, and the last section in the series is the most ventral (V). On each section through the brain, anterior is the bottom side of the section and posterior is at the top. For the sections through the ventral nerve cord in Figure 5B, the dorsalmost section is to the left and the ventralmost section is to the right; anterior (A) is at the top and posterior (P) is at the bottom. (A) Distribution of neurons labeled with the S1 probe in the brains of wild-type, fru^1, fru^2, and fru^4 (from left to right). Arrows indicate regions where fewer neurons associated with groups 3, 4, and 5 were found in fru^1 male brains. More heavily labeled neurons are present in all sections of fru^1 female brains when compared to fru^2, fru^3, and fru^4 females (open arrows point to regions where the additional neurons were most visible). The increase in the number of labeled neurons in fru^{sat} females was similar to that in fru^1 females.

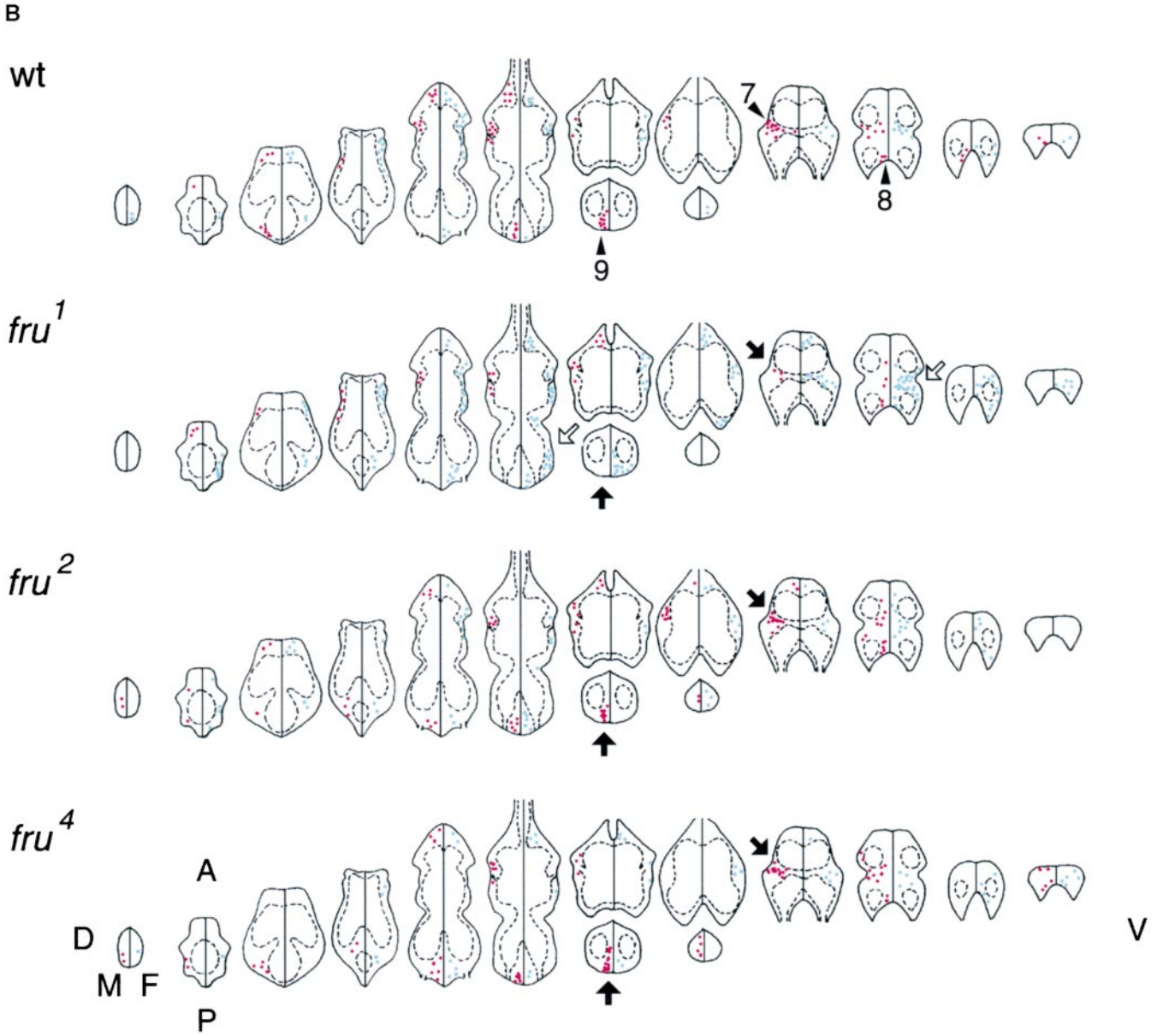

Figure 5.—(*Continued*). (B) Distribution of neurons in the ventral nerve cords of wild type, *fru*[1], *fru*[2], and *fru*[4] (from left to right). Solid arrows indicate regions in the abdominal ganglion (group 9) in which labeled neurons were not detected in *fru*[1], as well as cells in the thoracic ganglion (group 7), where fewer labeled neurons were found in *fru*[1] male ventral nerve cords compared to the other genotypes. More neurons were labeled in all sections of *fru*[1] female ventral nerve cords (examples indicated by open arrows) compared to females of all other genotypes.

was affected in the transposon mutants. In all four mutants, the S1 probe detected groups of labeled neurons in the nine locations where P1-expressing neurons are found in wild-type males (see the schematic representations in Figure 5, A and B; because no differences among the expression patterns for *fru*[2], *fru*[3], *fru*[4], and *fru*[sat] were detected, only the locations of labeled neurons for a *fru*[4] brain and thoracic/abdominal ganglia are shown). For example, labeled neurons were found in the antennal lobe (Figure 6, C–E, group 6), the dorsal anterior protocerebrum (Figure 6, H–J, groups 3 and 4), the thoracic ganglion (Figure 6, M and N, group 7), and the abdominal ganglion (Figure 6, Q and R, group 9). The numbers of neurons labeled in these groups were similar to those found in wild-type males (Table 2). However, there was an overall increase in the number of heavily labeled neurons in the brains of *fru*[sat] males compared to the other *fru* mutant or wild-type males (Table 4). In wild-type males, cells labeled with the S1 probe often had darkly stained dots within the nucleus, as well as cytoplasmic staining (*cf.* Ryner *et al.* 1996). In a similar fashion, in all the *P*-element-mutant males, neurons labeled with the S1 probe often had very darkly stained dots in the nucleus in addition to weak cytoplasmic staining (Figure 6, C–E, H–J, M, N, Q and R).

In situ hybridization to the four *P*-element mutants

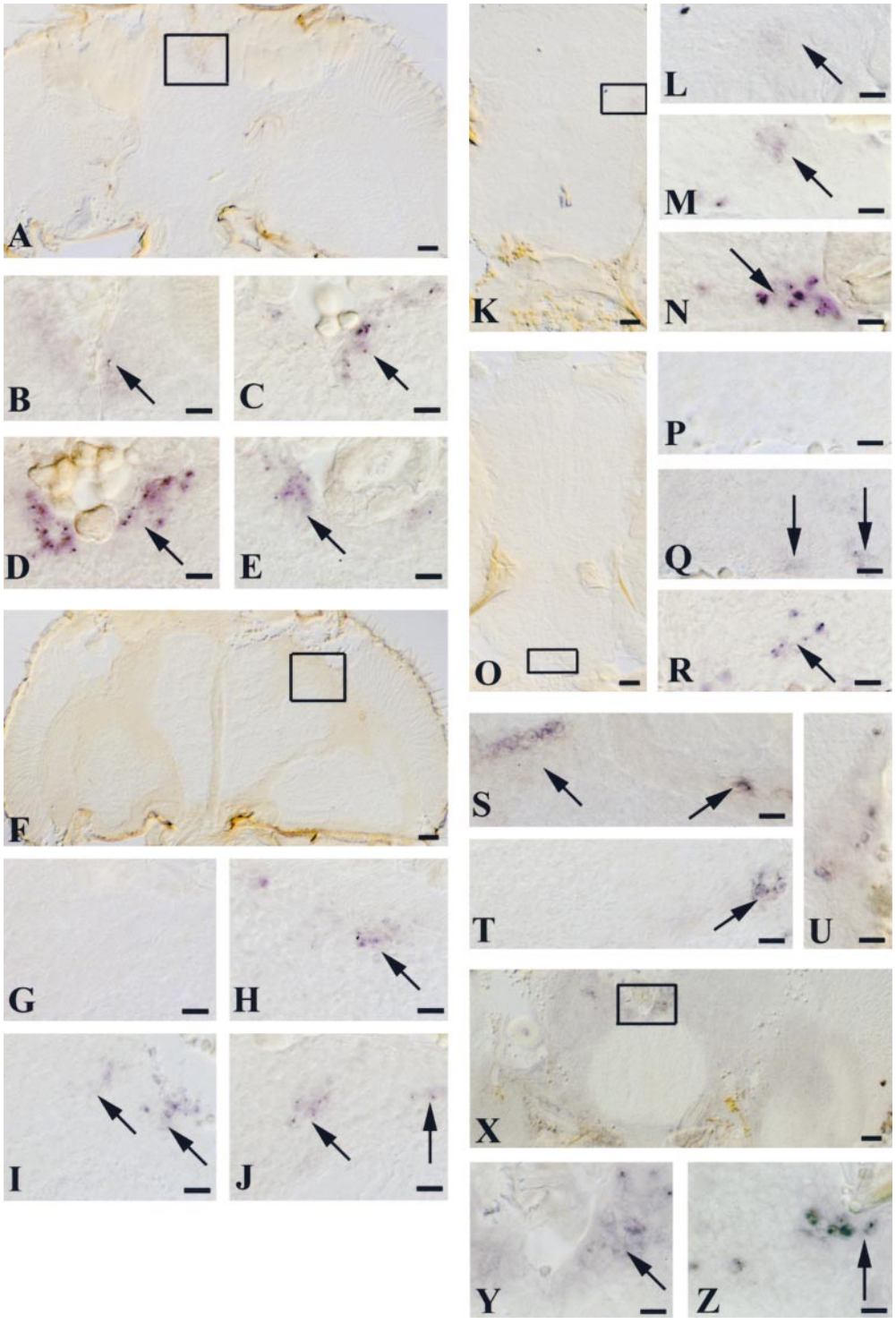

Figure 6.—Photomicrographs of *in situ* hybridizations in horizontal sections of *fruitless* mutants. (A–E) P1 promoter expression pattern in the mechanosensory part of the antennal lobe in males (*cf.* section 11 from the top in Figure 5A). Arrows in high-magnification views (B–E) point to labeled group-6 neurons (only cells on the right side are indicated, but both left and right sides are shown). (A) Low-magnification view of a fru^1 male head. (B) High-magnification view of the boxed region in A with labeled neurons (arrows) in the antennal lobe. (C) High-magnification view of a fru^2 male. (D) High-magnification view of a fru^3 male. (E) High-magnification view of a fru^4 male. (F–J) P1 promoter expression pattern in anterior lateral protocerebrum in males (*cf.* seventh section in Figure 5A). Arrows point to the labeled neurons in groups 3 and 4 in the dorsal anterior part of the protocerebrum. (F) Low-magnification view of the head of a fru^1 male. (G) High-magnification view of the boxed region in A; only one side of the protocerebrum is shown in the high-magnification image, and the midline is to the left in G–J. (H) High-magnification view of a fru^2 male. (I) High-magnification view of a fru^3 male. (J) High-magnification view of a fru^4 male. (K–N) P1 promoter expression pattern in the thoracic ganglia of males (*cf.* ninth section from the left in Figure 5B). Arrows point to labeled neurons in thoracic neuronal group 7 in L and M. (K) Low-magnification view of the thoracic ganglia in a fru^1 male. (L) High-power view of the boxed region in K. (M) High-magnification view of thoracic neurons in a fru^2 male. (N) High-magnification view of thoracic neurons in a fru^3 male. (O–R) P1 promoter expression pattern in the abdominal ganglion (*cf.* seventh section from the left in Figure 5B). Arrows point to labeled neurons in the abdominal neuronal group 9 in Q and R. (O) Low-magnification view of the ventral nerve cord of a fru^1 male. (P) High-magnification view of the boxed region in O of the abdominal ganglion, which has no labeled neurons. (Q) High-magnification view of the abdominal cluster of neurons in a fru^2 male. (R) High-magnification view of the abdominal cluster of neurons in a fru^3 male. (S–U) P1 promoter expression pattern in female *fru* CNS. (S) High-magnification view of labeled mechanosensory antennal lobe neurons (group 6; section 11 in Figure 5A); arrows point to neurons on both sides of the midline. (T) High-magnification view of labeled neurons (group 3; section 7 in Figure 5A) in the anterior lateral protocerebrum. (U) High-magnification view of the neurons labeled in the thoracic ganglia, showing relatively limited numbers of strongly labeled neurons and many nearby cells with moderate-to-light levels of staining; there are more labeled neurons in this region in fru^1 and fru^{sat} mutant females than in wild-type, fru^2, fru^3, or fru^4 mutant females. (X–Z) Labeled neurons in the brain and ventral nerve cord of fru^2 mutant males, detected by the common coding probe (C1). (X) Low-magnification view of the

TABLE 2

***fru*-expressing neurons in the male and female CNS of wild-type and *fruitless* mutants: numbers of neurons per hemi-CNS in wild-type (WT) and *fru*-mutant males labeled by *in situ* hybridization to P1 transcripts**

Male genotype		Group 1	Groups 3–5	Group 6	Group 7	Group 9
WT	(4)[a]	51.6 ± 8.4	45.5 ± 5.9	25.0 ± 5.9	28.8 ± 3.5	18.5 ± 0.6
fru^1	(3)	38.8 ± 3.3	11.5 ± 0.5[b]	21.0 ± 4.0	4.5 ± 0.8[c]	2.0 ± 1.5[d]
fru^2	(3)	35.5 ± 8.1	55.7 ± 10.3	16.8 ± 0.3	23.0 ± 5.5	13.2 ± 3.7
fru^3	(3)	55.1 ± 8.5	66.8 ± 16.8	26.1 ± 2.4	17.2 ± 4.6	12.2 ± 2.6
fru^4	(4)	45.1 ± 5.2	42.0 ± 7.5	21.5 ± 5.2	24.1 ± 5.1	14.5 ± 1.4
fru^{sat}	(5)	68.1 ± 6.3	50.5 ± 8.0	35.5 ± 2.9	30.1 ± 3.2	14.1 ± 1.6

Numbers ± SEMs are averages for neurons that were labeled with the S1 probe (see materials and methods) in CNS regions of Canton-S wild-type (WT) and *fru*-mutant males and females. Neurons that are part of the three groups in the anterior protocerebrum (groups 3–5) were counted as a single group. In females, neuronal groups in the brain were counted, because the male-specific groups 7 and 9 are not present. For all genotypes except fru^1 and fru^{sat} females, neurons were heavily labeled with the S1 probe and, thus, were distinguishable from neighboring unlabeled neurons. In fru^1 and fru^{sat} females, many neurons were labeled heavily; a new class of moderately and lightly labeled neurons, not found in animals of any other genotype, was also detected. For this analysis, only the heavily labeled neurons were counted. From the statistical analyses, all comparisons of mean values were not significant, except as indicated (see footnotes).

[a] Numbers (n) of late-stage pharate-adult specimens scored for each genotype or strain are in parentheses within this section and in Tables 3 and 4.

[b] The average numbers of neurons in groups 3–5 in fru^1 males are significantly different from those in fru^3 males.

[c] The average numbers of neurons in group 7 in fru^1 males are significantly different from those in wild-type, fru^4, and fru^{sat} males.

[d] The average numbers of neurons in group 9 in fru^1 males are significantly different from those in wild-type, fru^2, fru^3, fru^4, and fru^{sat} males.

with the C1 probe detected heavily labeled cells only in fru^2 males. These neurons were in the same positions as the nine groups labeled by the S1 probe (*e.g.*, group 6, *cf.* Figure 6, C and Y; group 7, *cf.* Figure 6, Z and M). These neurons had the same darkly stained dots in the nucleus, as was seen with the S1 probe (see above), in addition to having a higher level of cytoplasmic expression than the surrounding neurons (Figure 6, X and Y). Thus, the expression pattern in fru^2 males appears to be similar to that of wild-type males.

In contrast, in the CNS of fru^3, fru^4, and fru^{sat} males, no heavily expressing neurons were detected with the C1 probe, although there was a general low level of CNS staining. In these sections, only ~10 neurons in total had evidence of nuclear dots. These findings suggest that detectable levels of transcripts containing normal P1-derived transcripts are not found in the CNS neurons of fru^3, fru^4, and fru^{sat} males. Because a population of heavily labeled neurons was detected using the S1 probe in these mutants, the *fru*-expressing neurons are present, but it does not appear that intact P1 transcripts can be detected in these cells by *in situ* hybridization. This finding is in accord with the RT-PCR results presented above, in which it was necessary to use poly(A)$^+$ RNA from heads to detect normally spliced P1-derived transcripts in these mutants.

Using both the S1 and C1 probes, we also examined the effects of all five *fru* mutations on *fruitless* expression in females. In the four *P*-element mutants, the cellular distributions and expression levels of transcripts from the P1 promoter (Figure 5, A and B, S1 probe; Table 3) were comparable to those of wild-type females (data not shown). With the C1 probe, only fru^2 females showed heavily expressing neurons; these neurons were in the same locations as the cells detected by the S1 probe, suggesting that the same neurons are labeled with both probes (*cf.* Ryner *et al.* 1996). In contrast, in the CNS of fru^3, fru^4, and fru^{sat} females, no heavily expressing neurons were detected with the C1 probe (data not shown). This suggests that the levels of intact P1 tran-

mechanosensory antennal lobe (group 6; *cf.* section 12 in Figure 5A); the arrow points to the location of neurons with a relatively high level of expression; by their locations and relative number, these heavily labeled neurons are likely to be the same ones detected by the S1 probe; all other neuronal cell bodies in the section also show a low level of *fru* expression with this probe. (Y) High-magnification view of the boxed region indicated by arrow in A. (Z) High-magnification view of the thoracic group 7 (*cf.* ninth section in Figure 5B, showing that the male-specific groups of neurons in this region are also detected by probe C1 and show a relatively high level of staining). In all of these images, anterior is to the top. The scale bars for the low-magnification views are 100 μm and for the high-magnification ones are 20 μm.

TABLE 3

***fru*-expressing neurons in the male and female CNS of wild-type and *fruitless* mutants: Numbers of neurons per hemi-CNS in wild-type and *fru*-mutant females labeled by *in situ* hybridization to P1 transcripts**

Female genotype		Group 1	Groups 3–5	Group 6
WT	(4)	32.3 ± 3.8	77.5 ± 4.4	23.8 ± 4.0
fru^1	(4)	62.8 ± 7.7	87.3 ± 11.4	23.8 ± 3.6
fru^2	(3)	41.5 ± 12.8	105.3 ± 18.1	27.2 ± 8.7
fru^3	(3)	35.0 ± 7.4	54.0 ± 13.0	21.4 ± 4.3
fru^4	(2)	29.8 ± 7.3	62.8 ± 9.3	19.0 ± 1.5
fru^{sat}	(3)	52.2 ± 7.7	103.2 ± 12.1	32.0 ± 1.5

Numbers ± SEMs are averages for neurons that were labeled with the S1 probe (see materials and methods) in CNS regions of Canton-S wild-type (WT) and *fru*-mutant males and females. The neuronal groups indicated are those described in the text (see also Figure 5, A and B). Neurons that are part of the three groups in the anterior protocerebrum (groups 3–5) were counted as a single group. In females, neuronal groups in the brain were counted, because the male-specific groups 7 and 9 are not present. For all genotypes except fru^1 and fru^{sat} females, neurons were heavily labeled with the S1 probe and, thus, were distinguishable from neighboring unlabeled neurons. In fru^1 and fru^{sat} females, many neurons were labeled heavily; a new class of moderately and lightly labeled neurons that were not found in animals of any other genotype were also detected. For this analysis, only the heavily labeled neurons were counted. From the statistical analyses, all comparisons of mean values were not significant.

scripts are also below the level of detection by *in situ* hybridization in the neurons of fru^3, fru^4, and fru^{sat} females. Because a population of heavily labeled neurons is detected using the S1 probe, the *fru*-expressing neurons are present in these females, as they are in males of these genotypes.

Examination of the distribution of P1-derived transcripts (S1 probe) in fru^1 and fru^{sat} females revealed a substantial change in the distribution of labeled neurons. In fru^1 and fru^{sat} females, the overall staining intensity and number of labeled neurons were increased in the CNS compared to wild-type females (Figures 5, A and B, and 6, S–U; Table 3). The normal pattern of six groups of labeled neurons was still present in fru^1 and fru^{sat} female brains, and the numbers of labeled neurons were not significantly different from those in wild-type or other *P*-element mutants (Figure 5A; Table 3). The increase in the number of labeled neurons appeared in regions where labeled neurons are not found in wild-type males and females, *e.g.*, in ventral regions of the thoracic ganglion (Figures 5B and 6U). This increased expression of transcripts from the P1 promoter was confined to the CNS, because no expression was detected in the non-neural tissues of fru^1 and fru^{sat} females (data not shown). The difference between fru^1 and wild-type females in the numbers of heavily labeled neurons in the ventral thoracic CNS was statistically significant; although the numbers in the brain were also larger than normal, this difference was not statistically significant (Table 4).

When the C1 probe was used on fru^1 females, we observed heavily labeled neurons as well as widespread low-level neuronal expression in the CNS. As was found with the S1 probe, more neurons gave strong signals with the C1 probe in fru^1 than in wild-type females.

DISCUSSION

The primary purpose of this study was to gain insights into the molecular basis of the phenotypes of the five viable *fruitless* mutants, all of which result from lesions in noncoding regions of *fru* (Ito *et al.* 1996; Ryner *et al.* 1996). In addition, we have (1) extended what is known about the normal expression of this gene to provide a more robust framework for analyzing these mutants and (2) carried out a detailed study of the behavioral effects of fru^{sat} to put its phenotypic characterization on a par with that of the other viable mutants.

***fru* expression in wild-type:** Our analysis of normal *fruitless* expression focused on the transcripts produced by the P1 promoter, as we believe it is through the products of this promoter that *fru* controls male sexual behavior. Previous RT-PCR experiments showed that P1-derived transcripts are sex-specifically spliced near their 5′ end to generate male- and female-specific products (Ryner *et al.* 1996). Previous Northern analysis revealed three female-specific, three male-specific, and one common transcript in poly(A)$^+$ RNA from adult heads (Ryner *et al.* 1996). We suggested that the three male-specific and three female-specific mRNAs represented the P1-promoter-derived, male-specific, and female-specific 5′ ends spliced to each of the three alternative zinc-finger-encoding exons at the 3′ end of the *fru* ORF. Here, we have used additional probes to establish definitively that the three male and three female sex-specific transcripts are indeed all derived from the use of the P1 promoter, as predicted.

We also extended the analysis of wild type with respect to the distributions of P1-promoter-derived transcripts within the CNS (*cf.* Ryner *et al.* 1996). *In situ* hybridizations allowed the identification of nine major clusters

TABLE 4

***fru*-expressing neurons in the male and female CNS of wild-type and *fruitless* mutants: total number of labeled neurons from both sides of the brain and ventral nerve cord**

Genotype	Brain	Ventral nerve cord
Male		
WT	192.5 ± 22.5 (2)	194.0 ± 9.5 (3)
fru^{1}	204.5 ± 7.5 (2)	48.0 ± 6.0 (2)
fru^{2}	289.5 ± 46.5 (3)	157.3 ± 0.3 (3)
fru^{3}	296.0 ± 62.0 (3)	123.3 ± 38.4 (3)
fru^{4}	199.0 ± 42.0 (3)	216.0 ± 58.0 (3)
fru^{sat}	534.0 ± 35.9[a] (5)	234.6 ± 27.7[b] (3)
Female		
WT	383.6 ± 23.5 (3)	129.5 ± 9.1 (6)
fru^{1}	546.0 ± 51.9 (4)	368.0 ± 54.0[d] (5)
fru^{2}	430.0 ± 5.8 (3)	128.3 ± 18.9 (3)
fru^{3}	213.0 ± 45.5[c] (3)	58.3 ± 9.9 (3)
fru^{4}	376.0 ± 39.0 (3)	92.5 ± 39.5 (3)
fru^{sat}	617.0 ± 46.1 (3)	253.3 ± 14.4[e] (3)

Numbers ± SEMs are averages for neurons that were labeled with the S1 probe (see materials and methods) in CNS regions of Canton-S wild-type (WT) and *fru*-mutant males and females. The neuronal groups indicated are those described in the text (see also Figure 5, A and B). Neurons that are part of the three groups in the anterior protocerebrum (groups 3–5) were counted as a single group. In females, neuronal groups in the brain were counted, because the male-specific groups 7 and 9 are not present. For all genotypes except fru^{1} and fru^{sat} females, neurons were heavily labeled with the S1 probe and, thus, were distinguishable from neighboring unlabeled neurons. In fru^{1} and fru^{sat} females, many neurons were labeled heavily; a new class of moderately and lightly labeled neurons that were not found in animals of any other genotype were also detected. For this analysis, only the heavily labeled neurons were counted. From the statistical analyses, all comparisons of mean values were not significant, except as indicated (see footnotes).

[a] The average number of labeled neurons in the brains of fru^{sat} males is significantly different from the averages determined from scoring pharate-adult brains from males of all other genotypes tabulated.

[b] The average number of labeled neurons in the ventral nerve cord of fru^{sat} males is significantly different from the value determined by scoring the ventral nerve cord of fru^{1} males.

[c] The average number of labeled neurons in fru^{3} female brains is significantly different than those determined for fru^{1} and fru^{sat} female brains.

[d] The average number of labeled neurons in the ventral nerve cord of fru^{1} females is significantly different from values for the ventral nerve cords of Canton-S, fru^{2}, fru^{3}, and fru^{4} females.

[e] The average number of labeled neurons in the ventral nerve cord of fru^{sat} females is significantly different from that determined for the ventral nerve cord of fru^{3} females.

of cells in which the P1 promoter is expressed in the CNS and permitted the enumeration of the numbers of cells in each cluster. Thus, we specify in greater detail than previously (and now summarize diagrammatically) the brain and ventral-cord regions in which the sex-specific transcripts are found (Figure 5). These results led to a further consideration of CNS regions known from previous investigations to be involved in male courtship (see Introduction), including the most recent study of this kind (Ferveur and Greenspan 1998).

Comparing such behavioral analyses of genetic mosaics to the patterns of *fruitless* expression indicates that all CNS regions in which the P1 *fru* transcripts are found correspond to sites within the brain and the ventral nervous system whose male genotype is correlated with the performance of various steps within the courtship sequence.

Effects of *P*-element mutations on *fru* expression: Northern analysis, RT-PCR experiments, and *in situ* hybridization to tissue sections all led to a consistent view of the effects of the *P*-element *fru* alleles on gene expression.

Northern analysis showed that in the four *P*-element mutants, the normal array of six sex-specific P1 transcripts and one sex-nonspecific *fru* transcript was entirely absent; instead, novel arrays of transcripts were produced. Strikingly, the novel transcripts produced by fru^{3} and fru^{4} were equivalent, as were those observed in fru^{2} and fru^{sat}. Two further findings suggest that these novel arrays of transcripts come from the effects of these transposon inserts on the processing of *fruitless* transcripts. First, the transposons are all inserted in the large *fru* intron at locations well removed from the P1 promoter (Figure 1); our quantitative analysis of the cellular expression patterns of the P1 promoter in these mutants revealed no differences between these four mutants and wild type in the number or locations of cells expressing P1 transcripts. Second, the pairs of transposons that produced similar arrays of novel *fru* transcripts were either identical in terms of the sequences that they contained (fru^{3} and fru^{4}), or they shared some sequences in common (fru^{2} and fru^{sat}).

That effects of these mutations on the pattern of *fru* transcripts were due to sequences in these transposons providing splice-acceptor sites that are used in aberrant splicing events, as is known to occur for analogous transposon mutants in Drosophila (Horowitz and Berg 1995), were shown by RT-PCR analysis. Consistent with the Northern results (where six of the seven transcripts detected are produced by the P1 promoter), RT-PCR showed that in fru^{3} and fru^{4} mutants, transcripts from the P1 promoter were spliced to an acceptor site of the *l(3)S12* gene located in these transposons, whereas in fru^{2} and fru^{sat}, transcripts derived from P1 were spliced to the acceptor site of the *white* gene located in the latter transposons.

Additional RT-PCR experiments showed that the effects of fru^{2}, fru^{3}, fru^{4}, and fru^{sat} are not restricted to P1-derived transcripts. We found that transcripts from all *fru* promoters located 5′ to a given transposon insert (Figure 1) can be aberrantly spliced to an acceptor site

in the transposon, whereas splicing from *fru* promoters located 3′ to the insertion sites are unperturbed.

The findings that sequences in the transposons in the four *P*-element mutants lead to nonproductive splicing of *fru* transcripts offer a first-order explanation for how the insertions of these transposons into the large *fru* intron generate mutant phenotypes. However, what is not accounted for by these findings is that these four insertion mutations differ in the severity of their phenotypic effects (Table 1; Villella *et al.* 1997). One possible explanation for these phenotypic differences is that low levels of normal *fru* mRNAs are generated from the P1 promoter in these mutants. Indeed, RT-PCR experiments using poly(A)$^+$ RNA from heads were able to detect normally spliced products from the P1 promoter in all four of the transposon mutants. These RT-PCR experiments were not quantitative, allowing for the possibility that there are different residual levels of normal, P1-derived mRNAs in the mutants; this could account for the different severities of these alleles. That some of the transposons are not only intrinsically different, but also are inserted at different intronic positions, could contribute to the probability of a given transcript being subject to aberrant splicing during its processing.

Additional data bearing on the above points come from our *in situ* hybridizations to *fruitless* RNA. With respect to fru^3, fru^4, and fru^{sat}, these experiments also suggest that there is little normal splicing of P1 transcripts in these mutants: high levels of expression of *fru* common coding sequences were not detected in the cells in which the P1 promoter is expressed. There is a full complement of the neurons that normally express the P1 promoter in these three mutants despite the fact that there is little or no P1-promoter-derived mRNA that encodes *fru* proteins in these cells; this suggests that sex-specific *fru* expression is not necessary for the generation (during development) and subsequent survival of these cells in adults. A related point is that the expression of *fruitless*—in the context of *fru* mutation effects on behavior—may be involved in regulating the ongoing function of the nervous system in such flies. Indeed, all of our Northern analyses (Figures 2 and 3) involved detection of *fru* transcripts in mature adults.

In the case of fru^2, *in situ* hybridization detected high levels of expression of *fru* common coding sequences in the cells in which the P1 promoter is normally expressed, suggesting that there are significant levels of normally spliced P1-derived transcripts produced. This finding may account for the nearly normal behavioral phenotype of fru^2 males compared to the other *P*-element mutants (Villella *et al.* 1997). However, it should be noted that, while the *in situ* hybridization experiments revealed that both the P1-promoter-derived and *fru* common coding sequences are expressed at high levels in the same array of cells, such experiments did not establish that these sequences are in the same transcripts. This caveat is reinforced by the observation that Northern analysis with the common-region probe detected an abundant novel transcript class in fru^2 (Figure 3B), which was not seen with the probe to the P1 promoter region (Figure 3E).

One feature of the data from the *P*-element mutants that is not entirely accounted for are the specific arrays of novel transcripts detected on Northerns. In particular, if the only abnormal event in the processing of P1 transcripts in these mutants involves a splice from the male or female donor site to an acceptor site in the transposon, then a probe from the 5′ end of the P1 transcripts should detect one novel transcript in each sex. Whereas this is what was found in the cases of fru^2 and fru^{sat}, in the fru^3 and fru^4 mutants, more complex arrays of novel transcripts were detected by this probe. Possible explanations for the latter results are that these hybrid transcripts terminate at multiple sites, or that there are additional novel splices from the transposon sequences to sequences further downstream. If there are additional novel splicing events involved in the production of such RNAs, they are not to the normal acceptor site at the beginning of the *fru* common coding region, as a probe to that region does not detect these transcripts. Thus, whatever their exact nature, these RNA species are not likely to be a source of fru^+ function. In addition, it should be noted that a novel transcript of unknown origin is detected with the common region probe in the fru^2 mutant; whether this transcript encodes a functional *fru* product is unknown.

Behavioral effects of viable *fru* mutations: The molecular findings on the origins of *P*-element *fru* alleles should be considered in the context of phenotypic differences among these mutants. The effects of fru^1, fru^2, fru^3, and fru^4 on male sexual behaviors have been well characterized (Villella *et al.* 1997); here, we present comparable results for the courtship of fru^{sat} males. In contrast to what was reported previously, as a result of observations in which only wing extensions by the male were considered to be courtship actions (Ito *et al.* 1996; Yamamoto *et al.* 1996, 1997, 1998), we found that fru^{sat} and fru^{sat}/Df males exhibit appreciable courtship in the presence of females (Table 1). However, the behavior of fru^{sat} males is different from that of the other *P*-element *fruitless* variants (Villella *et al.* 1997). Of these mutations, fru^2 has the least severe effects on courtship: males homozygous for this allele court females vigorously, copulate, and are fertile. However, fru^2 males do not discriminate properly between males and females as courtship partners and show subtle abnormalities in their courtship song. fru^3 and fru^4 males are more severely affected: males homozygous for either of these mutations fail to discriminate between males and females as courtship partners, fail to generate any song pulses and do not attempt copulation. On the one hand, our analysis of fru^{sat} male behavior showed that the levels of courtship directed at flies of either sex (Table 1) are much less than observed for the other viable *fru* mutants. In this

regard, the behavioral effects of fru^{sat} are similar to those of chromosomal breakpoints that separate the P1 promoter from the *fru* ORF (Ryner *et al.* 1996; A. Anand, A. Villella, L. C. Ryner, T. Carlo, S. F. Goodwin, H-J. Song, J. C. Hall, B. S. Baker and B. J. Taylor, unpublished results). On the other hand, courtship chaining by fru^{sat} males (Table 1) occurred at levels comparable to those displayed by the other three *P*-element mutants (*cf.* Villella *et al.* 1997). Moreover, fru^{sat} males, like those expressing fru^{3} or fru^{4} (Villella *et al.* 1997), exhibit orientation, tapping, wing extension with no courtship pulse song, licking behavior, and no attempted copulation (Table 1). In this qualitative sense, fru^{sat} is similar in its behavior to the other two sterile transposon mutants; these three male types can be regarded as skipping a courtship step, with singing removed from the middle of the sequence. This suggests that not all the courtship steps occur in a dependent sequence.

The molecular results discussed above establish that the *P*-element alleles of *fruitless* are associated with high levels of aberrant splicing events in which *fru* sequences are spliced into sequences in the transposons. The associated reductions in the levels of wild-type *fru* transcripts are the likely cause of the mutant phenotypes in these mutants, and differences among these mutants in the severity of their phenotypes are likely due to differences in the levels of residual normal transcripts.

There is an alternative interpretation of certain aspects of the fru^{2} and fru^{sat} phenotypes that is worth commenting upon. These two mutants contain *white* genes in their transposon, and it has been shown that ectopic expression of the *white* gene product induces courtship among *D. melanogaster* males (Zhang and Odenwald 1995). Given that *fru-white* hybrid transcripts are produced in fru^{2} and fru^{sat} (see above), the *white* gene product could be expressed ectopically in behaviorally relevant brain neurons and be the cause of intermale courtships performed by these mutants. We think this interpretation is extremely unlikely because: (1) the chimeric transcript produced in these mutants encodes a protein containing FRU amino acids fused out-of-frame with *white* sequences, and (2) the fru^{2} and fru^{sat} mutations are recessive for intermale chaining behavior (Gailey and Hall 1989; Ito *et al.* 1996; Villella *et al.* 1997; this study), whereas ectopic expression of w^{+} is dominant with respect to its effects on courtship (Zhang and Odenwald 1995; Hing and Carlson 1996). Consequently, the behavioral anomalies of these two *fruitless* mutants probably result from subnormal levels of *fru* transcripts.

Effects of fru^{1} mutation on *fruitless* expression: The fru^{1} mutation does not cause any detectable change in the array of *fru* transcripts. However, fru^{1} exhibits changes in the numbers of neurons expressing detectable levels of P1-derived transcripts. In fru^{1} males, there are marked reductions in the numbers of cells expressing P1-derived transcripts in five of the nine groups of cells in which these transcripts are expressed in the wild type. The groups affected include three groups in the anterior protocerebrum, a male-specific neuronal group in the thoracic ganglia, and a male-specific neuronal group in the abdominal ganglia. In the remaining four groups, there were normal numbers of neurons expressing P1-derived transcripts. That the expression pattern affected by fru^{1} involves neurons in only certain regions of the CNS leads us to suggest that the behaviors defective in this mutant are controlled, at least in part, by specific subsets of the neurons expressing P1-derived transcripts. In particular, the reduction in the number of *fruitless*-expressing neurons in a thoracic-ganglionic cluster of fru^{1} males may account for the defect in fru^{1}'s singing behavior (*e.g.*, Wheeler *et al.* 1989). Other fru^{1} behaviors, such as wing extension, are normal, and the dorsal-posterior brain region linked to this behavior (Hall 1977, 1979) is unaffected by this mutation in terms of *fru* expression. Furthermore, the absence of *fru*-expressing neurons in the abdominal-ganglion neuronal group in fru^{1} may underlie the associated defect in attempted copulation (*cf.* Hotta and Benzer 1976; Hall 1977, 1979). On the basis of these results, we propose that *fru*-labeled neurons in the anterior regions of the dorsal protocerebrum contribute to the control of courtship-partner recognition, and the male-specific cell clusters in the thoracic CNS may be neural substrates for courtship song (singing has been mapped to the thoracic CNS; Schilcher and Hall 1979; Hall *et al.* 1990). Consistent with these notions are the identification, by higher-resolution mosaic analysis, of newly appreciated CNS regions involved in attempted copulation and copulation (Ferveur and Greenspan 1998).

On the control of male sexual behavior by *fru*: We previously proposed that the male-specific products of the P1 *fru* promoter are responsible for establishing the potential for male sexual behaviors (Ryner *et al.* 1996). Two kinds of observations supported this suggestion. First, male sexual behavior is negatively regulated by the action of the sex-determination hierarchy through functioning of the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes. In females, *tra* and *tra-2* regulate the sex-specific splicing of the transcripts derived from the *doublesex* (*dsx*) gene and the P1 *fru* promoter to generate female-specific products from these genes; in males, default splicing generates male-specific *dsx* and *fru* products. Because *dsx* mutants affect relatively few aspects of male sexual behavior (Villella and Hall 1996), whereas *fru* mutations affect nearly all aspects of the male-performed sequence, we suggest that the P1 *fru* transcripts function to establish the potential for male sexual behavior. Second, characterization of the cellular expression pattern of the P1 *fru* promoter revealed that it is expressed in only a very restricted number of CNS cells, and P1-expressing cells are in regions of the CNS

that previous studies have implicated in particular aspects of male sexual behavior.

Two features of our findings with respect to the viable *fruitless* mutants are relevant to the idea that the P1 *fru* products govern male sexual behavior. First, the finding that all four of the *P*-element alleles we have analyzed result in reduced levels of wild-type P1 transcripts is consistent with the suggested role of these transcripts in male behavior. However, the additional findings that each of these alleles also interferes with the processing of transcripts from other *fru* promoters make it likely that there are reduced levels of wild-type products from these promoters. This raises the question of whether the products of these other promoters are needed in conjunction with the products of the P1 promoter to establish the potential for male sexual behavior. Second, our results showing that fru^1 leads to the absence of P1-derived transcripts in a subset of the CNS cells in which the P1 transcripts are normally expressed provide compelling evidence for the role of P1-derived transcripts in male sexual behavior. Moreover, these data also provide strong support for the notion that the cells expressing P1 transcripts in the CNS, as identified by *in situ* hybridization, govern male sexual behavior.

We thank Daisuke Yamamoto for the fru^{sat} stock, Gail Fasciani and Julia Becker for helping with fly collections, Celeste Berg for providing the p*PZ* plasmid, Becky Meyers for automated sequencing, Edward Dougherty for photographic assistance, Laura Wilson and Randy Bender for *in situ* hybridizations, and Frank Moore for access to a cryostat. We appreciate comments on the manuscript from Ralph J. Greenspan, Troy Carlo, Michael Rosbash, Jean-Christophe Billeter, Megan C. Neville, Ravi Allada, and Donald A. Gailey. This work was supported by National Institutes of Health grants NS-33252 and GM-21473. Current financial support to S. F. Goodwin, from the Wellcome Trust and Division of Molecular Genetics, University of Glasgow, is gratefully acknowledged.

LITERATURE CITED

Albagli, O., P. Dhordain, C. Deweindt, G. Lecocq and D. Leprince, 1995 The BTB/POZ domain: a new protein-protein interaction motif common to DNA- and actin-binding proteins. Cell Growth Differ. **6:** 1193–1198.

Castrillon, D. H., P. Gönczy, S. Alexander, R. Rawson, C. G. Eberhart *et al.*, 1993 Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single *P*-element mutagenesis. Genetics **135:** 489–505.

Cobb, M., and J-F. Ferveur, 1996 Evolution and genetic control of mate recognition and stimulation in *Drosophila*. Behav. Process. **35:** 35–54.

Ferveur, J-F., and R. J. Greenspan, 1998 Courtship behavior of brain mosaics in *Drosophila*. J. Neurogenet. **12:** 205–226.

Gailey, D. A., and J. C. Hall, 1989 Behavior and cytogenetics of *fruitless* in *Drosophila melanogaster*: different courtship defects caused by separate, closely linked lesions. Genetics **121:** 773–785. [corrigendum: Genetics **122:** 465 (1989)].

Gailey, D. A., B. J. Taylor and J. C. Hall, 1991 Elements of the *fruitless* locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of *Drosophila melanogaster* adults. Development **113:** 879–890.

Goodwin, S. F., M. Del Vecchio, K. Velinzon, C. Hogel, S. R. H. Russell *et al.*, 1997 Defective learning in mutants of the *Drosophila* gene for a regulatory subunit of cAMP-dependent protein kinase. J. Neurosci. **17:** 8817–8827.

Hall, J. C., 1977 Portions of the central nervous system controlling reproductive behavior in *Drosophila melanogaster*. Behav. Genet. **4:** 291–312.

Hall, J. C., 1979 Control of male reproductive behavior by the central nervous system of Drosophila: dissection of a courtship pathway by genetic mosaics. Genetics **92:** 437–457.

Hall, J. C., 1994 The mating of a fly. Science **264:** 1702–1714.

Hall, J. C., S. J. Kulkarni, C. P. Kyriacou, Q. Yu and M. Rosbash, 1990 Genetic and molecular analysis of neural development and behavior in *Drosophila*, pp. 100–112 in *Developmental Behavior Genetics*, edited by M. E. Hahn, J. K. Hewitt, N. D. Henderson and R. Benno. Oxford University Press, New York.

Hazelrigg, T., R. Levis and G. M. Rubin, 1984 Transformation of white locus DNA in Drosophila: dosage compensation, zeste interaction, and position effects. Cell **36:** 469–481.

Heinrichs, V., L. C. Ryner and B. S. Baker, 1998 Regulation of sex-specific selection of *fruitless* 5′ splice sites by *transformer* and *transformer-2*. Mol. Cell. Biol. **18:** 450–458.

Hing, A., and J. R. Carlson, 1996 Male-male courtship behavior induced by ectopic expression of the *Drosophila white* gene: role of sensory function and age. J. Neurobiol. **30:** 454–464.

Horowitz, H., and C. A. Berg, 1995 Aberrant splicing and transcription termination caused by *P*-element insertion into the intron of a Drosophila gene. Genetics **139:** 327–335.

Hotta, Y., and S. Benzer, 1976 Courtship in *Drosophila* mosaics: sex-specific foci for sequential action patterns. Proc. Natl. Acad. Sci. USA **73:** 4154–4158.

Hu, S., D. Fambrough, J. R. Atashi, C. S. Goodman and S. T. Crews, 1995 The *Drosophila abrupt* gene encodes a BTB-zinc finger regulatory protein that controls the specificity of neuromuscular connections. Genes Dev. **9:** 2936–2948.

Ito, H., K. Fujitani, K. Usui, K. Shimizu-Nishikawa, S. Tanaka *et al.*, 1996 Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. Proc. Natl. Acad. Sci. USA **93:** 9687–9692.

Mlodzik, M., and Y. Hiromi, 1992 The enhancer trap method in Drosophila: its application to neurobiology. Methods Neurosci. **9:** 397–414.

Moses, K., M. C. Ellis and G. M. Rubin, 1989 The *glass* gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. Nature **340:** 531–536.

O'Connell, P., and M. Rosbash, 1984 Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. Nucleic Acids Res. **12:** 5495–5513.

Pepling, M., and S. M. Mount, 1990 Sequence of a cDNA from the *Drosophila* melanogaster *white* gene. Nucleic Acids Res. **18:** 1633.

Read, D., and J. L. Manley, 1992 Alternatively spliced transcripts of the *Drosophila tramtrack* gene encode zinc finger proteins with distinct DNA binding specificities. EMBO J. **11:** 1035–1044.

Read, D., M. Levine and J. L. Manley, 1992 Ectopic expression of the *Drosophila tramtrack* gene results in multiple embryonic defects, including repression of even-skipped and *fushi tarazu*. Mech. Dev. **38:** 183–195.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella *et al.*, 1996 Control of male sexual behavior and sexual orientation in Drosophila by the *fruitless* gene. Cell **87:** 1079–1089.

Sagerström, C. G., and H. L. Sive, 1996 RNA blot analysis, pp. 83–103 in *A Laboratory Guide to RNA, Isolation, Analysis, and Synthesis*, edited by P. A. Krieg. Wiley-Liss, New York.

Schilcher, F. V., and J. C. Hall, 1979 Neural topography of courtship song in sex mosaics of *Drosophila melanogaster*. J. Comp. Physiol. **129:** 85–95.

Taylor, B. J., A. Villella, L. C. Ryner, B. S. Baker and J. C. Hall, 1994 Behavioral and neurobiological implications of sex-determining factors in *Drosophila*. Dev. Genet. **15:** 275–296.

Tower, J., G. H. Karpen, N. Craig and A. C. Spradling, 1993 Preferential transposition of *Drosophila P* elements to nearby chromosomal sites. Genetics **133:** 347–359.

Villella, A., and J. C. Hall, 1996 Courtship anomalies caused by *doublesex* mutations in *Drosophila melanogaster*. Genetics **143:** 331–344.

Villella, A., D. A. Gailey, B. Berwald, S. Ohshima, P. T. Barnes

et al., 1997 Extended reproductive roles of the *fruitless* gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics **147:** 1107–1130.

von Kalm, L., K. Crossgrove, D. Von Seggern, G. M. Guild and S. K. Beckendorf, 1994 The Broad-Complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. EMBO J. **13:** 3505–3516.

Wheeler D. A., S. J. Kulkarni, D. A. Gailey and J. C. Hall, 1989 Spectral analysis of courtship songs in behavioral mutants of *Drosophila melanogaster*. Behav. Genet. **19:** 503–528.

Wilson C., R. K. Pearson, H. J. Bellen, C. J. O'Kane, U. Grossniklaus *et al.*, 1989 *P*-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. Genes Dev. **3:** 1301–1313.

Yamamoto, D., H. Ito and K. Fujitani, 1996 Genetic dissection of sexual orientation: behavioral, cellular, and molecular approaches in *Drosophila melanogaster*. Neurosci. Res. **26:** 95–107.

Yamamoto, D., J. M. Jallon and A. Komatsu, 1997 Genetic dissection of sexual behavior in *Drosophila melanogaster*. Annu. Rev. Entomol. **42:** 551–585.

Yamamoto, D., K. Fujitani, K. Usui, H. Ito and Y. Nakano, 1998 From behavior to development: genes for sexual behavior define the neuronal sexual switch in *Drosophila*. Mech. Dev. **73:** 135–146.

Zhang, S.-D., and W. F. Odenwald, 1995 Misexpression of the *white* gene triggers male-male courtship in *Drosophila*. Proc. Natl. Acad. Sci. USA **92:** 5525–5529.

Communicating editor: T. Schüpbach