Molecular Genetic Dissection of the Sex-Specific and Vital Functions of the *Drosophila melanogaster* Sex Determination Gene *fruitless*

Anuranjan Anand,*,† Adriana Villella,‡ Lisa C. Ryner,* Troy Carlo,‡ Stephen F. Goodwin,‡,§ Ho-Juhn Song, Donald A. Gailey,†† Ana Morales,‡ Jeffrey C. Hall,‡ Bruce S. Baker* and Barbara J. Taylor****

***Department of Zoology, Oregon State University, Corvallis, Oregon 97331-2914, ‡Department of Biology, Brandeis University, Waltham, Massachusetts 02254, *Department of Biological Sciences, Stanford University, Stanford, California 94305, †Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560 064, India , §Division of Molecular Genetics, University of Glasgow, Glasgow G11 6NU, United Kingdom and ††Department of Biological Sciences, California State University, Hayward, California 94542*

Manuscript received September 29, 2000
Accepted for publication May 10, 2001

ABSTRACT

A multibranched hierarchy of regulatory genes controls all aspects of somatic sexual development in *Drosophila melanogaster*. One branch of this hierarchy is headed by the *fruitless (fru)* gene and functions in the central nervous system, where it is necessary for male courtship behavior as well as the differentiation of a male-specific abdominal structure, the muscle of Lawrence (MOL). A preliminary investigation of several of the mutations described here showed that the *fru* gene also has a sex-nonspecific vital function. The *fru* gene produces a complex set of transcripts through the use of four promoters and alternative splicing. Only the primary transcripts produced from the most distal (P1) promoter are sex-specifically spliced under direction of the sex-determination hierarchy. We have analyzed eight new *fru* mutations, created by X-ray mutagenesis and *P*-element excision, to try to gain insight into the relationship of specific transcript classes to specific *fru* functions. Males that lack the P1-derived *fru* transcripts show a complete absence of sexual behavior, but no other defects besides the loss of the MOL. Both males and females that have reduced levels of transcripts from the P3 promoter develop into adults but frequently die after failing to eclose. Analysis of the morphology and behavior of adult escapers showed that P3-encoded functions are required for the proper differentiation and eversion of imaginal discs. Furthermore, the reduction in the size of the neuromuscular junctions on abdominal muscles in these animals suggests that one of *fru*'s sex-nonspecific functions involves general aspects of neuronal differentiation. In mutants that lack all *fru* transcripts as well as a small number of adjacent genes, animals die at an early pupal stage, indicating that *fru*'s function is required only during late development. Thus, *fru* functions both in the sex-determination regulatory hierarchy to control male sexual behavior through sex-specific transcripts and sex-nonspecifically to control the development of imaginal discs and motorneuronal synapses during adult development through sex-nonspecific transcript classes.

MULTIPLE promoters, alternative splicing, and complex sets of *cis*-acting sequences governing temporal and spatial patterns of gene expression are all employed to allow single genes to carry out multiple unrelated biological functions. The *fruitless* (*fru*) gene of *Drosophila melanogaster* is a particularly interesting gene that is representative of such multifunctional genes. *fru* has been shown to function as a member of the Drosophila sex determination regulatory hierarchy (Ryner *et al.* 1996; Heinrichs *et al.* 1998), where it acts to build the potential for male sexual behaviors into the male central nervous system (Ito *et al.* 1996; Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000; Lee *et al.* 2000; Usui-Aoki *et al.* 2000). In addition, *fru* function is essential for the viability of both sexes (Ryner *et al.* 1996).

Corresponding author: Bruce S. Baker, Department of Biological Sciences, Stanford University, Stanford, CA 94305.
E-mail: bbaker@cmgm.stanford.edu

The multiple functions of the *fru* gene are encoded by a complex set of sex-specific and sex-nonspecific transcripts that are generated by the use of four promoters (P1–4) and alternative splicing at both the 5′ and 3′ ends of the primary transcripts (Ito *et al.* 1996; Ryner *et al.* 1996; Goodwin *et al.* 2000; Usui-Aoki *et al.* 2000; L. C. Ryner, S. F. Goodwin, T. Carlo, M. Foss, J. C. Hall, B. J. Taylor and B. S. Baker, unpublished results). The transcripts from each of *fru*'s promoters have long open reading frames that encode closely related proteins belonging to the BTB-Znf family (*cf.* Albagli *et al.* 1995; Hu *et al.* 1995). Transcripts generated from the distal (P1) *fru* promoter are spliced sex-specifically under the control of transformer (TRA) and transformer-2 (TRA-2) proteins in females, whereas in males a default splice occurs (Ryner *et al.* 1996; Heinrichs *et al.* 1998). These sex-specifically spliced P1 transcripts are expressed in a small subset of neurons in the central nervous system (CNS; Ryner *et al.* 1996; Lee *et al.* 2000; Usui-Aoki *et al.* 2000) and are likely responsible for *fru*'s

role in male sexual behavior. In females, the splicing of the P1-derived transcripts gives rise to mRNAs with the potential to encode proteins with a BTB domain at their amino termini and one of three alternative Zn finger pairs at their carboxy termini (Goodwin *et al.* 2000; L. C. Ryner, S. F. Goodwin, T. Carlo, M. Foss, J. C. Hall, B. J. Taylor and B. S. Baker, unpublished results). In males, the default splicing of the P1 transcripts generates male-specific mRNAs, encoding proteins that differ from the proteins predicted to be produced from the female mRNAs by the addition of 101 amino acids N-terminal to the BTB domain. Strikingly, immunohistochemistry with anti-FRU antibodies shows that the mRNAs produced from the P1 *fru* promoter are not translated in females, whereas they are translated in males (Lee *et al.* 2000; Usui-Aoki *et al.* 2000). The transcripts from the three more proximal promoters (P2, P3, and P4) encode sex-nonspecific proteins that differ from the male-specific proteins by having just short stretches of amino acids preceding the BTB domain (L. C. Ryner, S. F. Goodwin, T. Carlo, M. Foss, J. C. Hall, B. J. Taylor and B. S. Baker, unpublished results). The roles of these sex-nonspecific proteins are not known, but it has been suggested that one or more of them are responsible for *fru*'s vital function (Ryner *et al.* 1996). Moreover, expression data show that one or more of these products are expressed widely in the nervous system as well as in some other tissues in both sexes (Ryner *et al.* 1996; Lee *et al.* 2000).

Most of the *fru* mutations or mutant combinations studied to date have been viable hypomorphs (Ito *et al.* 1996; Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000). Nevertheless, these studies have revealed that wild-type *fru* function is required for most steps in male courtship behavior but has no detectable role in female courtship behavior. *fru* males typically display reduced levels of courtship to females, as measured by the amount of time that a male courts a prospective mate (Hall 1978; Gailey and Hall 1989; Gailey *et al.* 1991; Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000). In addition, males of many *fru* genotypes are defective in specific parts of the courtship sequence (Gailey *et al.* 1991; Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000). For example, wild-type males choose females rather than males as the appropriate sexual partner, while *fru* males fail to distinguish between the sexes and as a consequence court females and males roughly equally (Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000). Indeed, when *fru* males are housed together, male-male courtship leads to the formation of groups of courting males (Hall 1978; Gailey and Hall 1989; Gailey *et al.* 1991; Ito *et al.* 1996; Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000). At a slightly later stage of courtship, when wild-type males extend a wing and vibrate it to produce a courtship song, certain *fru* mutants—for example, fru^{3}, fru^{4}, and fru^{sat} males—rarely extend a wing toward females and do not produce courtship pulse song during the short bouts of wing extension that they produce (Ryner *et al.* 1996; Villella *et al.* 1997). Copulation is not attempted by males of most *fru* genotypes, rendering these males sterile (but see Gailey *et al.* 1991;Villella *et al.* 1997; Goodwin *et al.* 2000). Finally, it was recently found by examining *fru* mutant combinations that do copulate that behaviors during mating, such as transfer of seminal fluids and sperm and the duration of mating, are also governed by *fru* (Lee *et al.* 2001). While the *fru* mutations characterized in these studies have strong effects on male courtship behaviors, the full phenotypic consequence of removing *fru* function has not been established.

To better understand *fru*'s functions, we conducted mutageneses of existing *fru P*-element alleles with the hope of identifying loss-of-function *fru* alleles and alleles that removed subsets of *fru*'s transcript classes. Eight new lesions were obtained in the *fru* locus. Our initial analysis of some of these mutants (Ryner *et al.* 1996) showed that *fru's* role in controlling male-specific behavior was more extensive than had been previously recognized and that *fru* had a vital function in both sexes (see also Ito *et al.* 1996). From analyzing male courtship behaviors in numerous mutant combinations, we show here that *fru* is involved in every step of the courtship ritual, thus extending the role of *fru* to encompass all aspects of male courtship. We analyzed in detail the anatomical, neuronal, and behavioral phenotypes associated with these new lethal *fru* lesions and combinations of these lesions with preexisting mutations and deficiencies of *fru*. Among the new anatomical phenotypes seen in lethal *fru* mutants were defects in the adult derivatives of the imaginal discs and a reduction in the terminal arborization of motor axons on adult abdominal muscles, suggesting that *fru* plays a role in the differentiation of the adult epidermis and CNS. In addition, we determined the effects of the new mutations on the expression of the different *fru* transcripts and correlated *fru*'s sex-specific and sex-nonspecific functions with the expression of particular classes of transcripts. Our results establish that *fru* functions sex-specifically in the sex-determination regulatory hierarchy to control male sexual behavior and sex-nonspecifically to control the development of imaginal discs and motorneuronal synapses during adult development.

MATERIALS AND METHODS

Generation of new *fru* mutations: *Fly strains and crosses:* Flies were reared at 25° in 70% relative humidity on a standard cornmeal, yeast, molasses (or dextrose), and agar diet with proprionic acid and/or tegosept added to inhibit mold.

Previously outcrossed stocks of $fru^{1,4}$ (for details see Goodwin *et al.* 2000) were balanced with *TM3, Sb ry, MKRS, Sb ry, TM6C, Sb Tb,* or *TM6B, Hu Tb e* balancer chromosomes (for details on these markers, see Lindsley and Zimm 1992). The fru^{2} allele was recently reextracted by crossing to a "Can-

tonized" *white* stock. The fru^{sat} and $Df(3R)fru^{sat15}$ alleles (Ito *et al.* 1996; kindly provided by D. Yamamoto) were maintained over *TM6B* and *TM6C* balancer chromosomes, respectively. Crosses with third chromosomal deficiencies that have breakpoints in, or close to, the *fru* locus, $Df(3R)Cha^{M5}$, $Df(3R)Cha^{M7}$, *Df(3R)P14*, *Df(3R)BX5*, $Df(3R)gl^{BX7}$, and *Df(3R)148.5-1* (Table 1 and Figure 1A; Gailey and Hall 1989; Gailey *et al.* 1991), were used in conjunction with the newly generated *fru* mutations to analyze mutant phenotypes.

Mutageneses: To generate more severely mutant derivatives of the fru^2 allele, which is due to the insertion of a *P* element ($p^p[wA]$) in the *fru* locus, fru^2 homozygous males were X rayed (4000 R, 115 V, 5 mA, 1-mm Plexiglas filter, Torrex X-ray machine) and crossed to *w; Dr Pr/TM3, Sb ry* virgin females. From 62,000 progeny, 31 white-eyed flies that had lost the w^+ marker of the *P* element in *fru* were obtained. A total of 16 lines were established by backcrossing three times to *w; Dr Pr/TM3, Sb ry* flies; lines were not established from the other 15 animals. These 16 lines were tested for sterility over the fru^2 parental chromosome. Seven new *fru* alleles, fru^{w9}, fru^{w10}, fru^{w12}, fru^{w13}, fru^{w24}, fru^{w25}, and fru^{w27}, were identified on the basis of the fact that these chromosomes were weakly fertile or sterile over fru^2 (Table 4). The remaining nine chromosomes were fertile over fru^2, cytologically normal in the *fru* region, and were not studied further.

Excisions of the fru^4 *P* element (*P[lacZ; ry*$^+$*]*; Castrillon *et al.* 1993) were generated using a standard transposase source (Robertson *et al.* 1988). Of the 90 ry^- lines generated from the remobilization of the fru^4 *P* element, Southern analysis showed that 37 were precise, or nearly precise, excisions of the *P* element, 23 were small deletions, and 3 were lethal. One of the lethal mutations, $Df(3R)fru^{4\text{-}40}$, which is lethal over $Df(3R)Cha^{M5}$ but not over *Df(3R)P14* (Table 3), was used in this study.

Molecular characterization of the new *fru* alleles: *Determining the nature of their chromosomal rearrangements:* To determine the type of lesion present in the new *fru* mutants, cytological analysis was done on salivary chromosomes from $fru^-/+$ third instar larvae.

Southern analyses were carried out on $fru^{w9}/TM3$, $fru^{w12}/TM3$, and $fru^{w27}/TM3$ adults to localize the position of these chromosomal breakpoints. We used labeled *P*^{p}*[wA]* DNA (Hazelrigg *et al.* 1984; Flybase segment ID no. FBmc0000129) to probe Southern blots of restriction digests of genomic DNAs of the heterozygous *fru* mutants and control genomic DNAs obtained from flies of the parental stock used in the mutagenesis, $fru^2/TM3$, and flies heterozygous for a complete deficiency of the region, $Df(3R)fru^{w24}/TM3$.

$Df(3R)fru^{4\text{-}40}$ was mapped using quantitative Southern blots of genomic DNA from mutant heterozygotes, wild-type control, homozygous fru^4 control, and balancer control flies probed with a series of phage probes (f3A, f2A, f1H, f4B, and f5C) spanning the *fru* locus (see Figure 1A, Ryner *et al.* 1996). These experiments showed that $Df(3R)fru^{4\text{-}40}$ has one end in the fru^4 *P* element and extends distally beyond the P1 promoter.

$Df(3R)fru^{sat15}$ was roughly mapped by a combination of Southern blot analysis and genomic PCR using oligo primers derived from plasmid subclones from across *fru*. To determine the exact endpoints of the deficiency, oligonucleotides flanking the breakpoints were utilized with PCR to amplify a fragment containing the sequences flanking the breakpoints. Sequencing of this product and comparison to the genomic sequence for this region showed that the deletion begins 20 nucleotides into the inverted repeat of the fru^{sat} *P* element and deletes all nucleotides from 96,551 to 13,839 of ACC no. 003722, deleting the entire coding sequence of *fru* (data not shown). In addition, $Df(3R)fru^{sat15}$ completely deletes the four transcription units immediately proximal to *fru* as well as parts of two overlapping transcription units that are the fifth and sixth transcription units proximal to *fru*.

Transcript analysis by reverse transcription PCR: To determine which *fru* transcripts were produced by various *fru* alleles, the following genotypes were analyzed: $Df(3R)fru^{w24}/T(3;het)fru^{w12}$, $Df(3R)fru^{w24}/In(3R)fru^{w27}$, $T(3;het)fru^{w12}/In(3R)fru^{w27}$, $Df(3R)fru^{4\text{-}40}/Df(3R)fru^{sat15}$, and $Df(3R)Cha^{M5}/T(3;het)fru^{w12}$. Since most of these genotypes die before adulthood, we collected ~50 *fru* mutant animals prior to their lethal phase, which was 1- to 2-day-old pupae for $Df(3R)fru^{w24}/T(3;het)fru^{w12}$, $Df(3R)fru^{w24}/In(3R)fru^{w27}$, $T(3;het)fru^{w12}//In(3R)fru^{w27}$ and adult flies for $Df(3R)fru^{4\text{-}40}/Df(3R)fru^{sat15}$ and $Df(3R)Cha^{M5}/T(3;het)fru^{w12}$. Each of these genotypes was generated from crosses using stocks balanced with the *TM6B, Hu Tb e* balancer. Animals were quick frozen and stored at −80°. Total RNA was extracted using the TRIzol reagent (GIBCO-BRL, Grand Island, NY). Reverse transcription was performed on total RNA from an equivalent of ~2.5 animals for a given genotype using a primer complementary to a sequence from a region common to all known *fru* transcripts [primer (fru-8-rev) 5′ gtgagaccacgcacctgtgcag-3′]. One-tenth of the reverse transcription reactions were used for the PCRs. Six different primer sets were used for the PCR to generate products diagnostic of the following: (A) transcripts containing the common coding portion of *fru* [primers 5′-aacact gaccaaggagcgatg-3′ (fru-25) and 5′-atgggcagcgaactctggcc-3′ (fru-26-rev)]; (B) transcripts from the P1 promoter spliced in the female pattern [first round primers 5′-ccagatcgaaagagaa tatcatca-3′ (fru-2) and 5′-cagcgcaagcagaattgctgg-3′ (fru-7-rev); second round primers 5′-taattctaaccgaaagtaagcatag-3′ (fru-32) and fru-7-rev]; (C) *fru* transcripts from the P1 promoter spliced in the male pattern [primers 5′-cttccgcccgcatcccctag-3′ (fru-31) and fru-26-rev]; note that this primer pair also amplifies the female-specific product from this region; (D) transcripts initiated from the P2 promoter [primers: both rounds forward primer 5′-atcataaaatcgctcggttttagtt-3′ (fru-29); first round reverse primer fru-26-rev; second round reverse primer 5′-catgaa ctcgagcagagatcgca-3′ (fru-55-rev-Xho)]; (E) transcripts initiated from the P3 promoter [first round forward primer 5′-ctgagaacgtgcgcgagtgtt-3′ (fru-11); second round forward primer 5′-caaagtgagtgagatacaatcgc-3′ (fru-12); reverse primer used in both rounds fru-7-rev]; (F) transcripts initiated from the proximal-most *fru* promoter, P4 [primers 5′-ttacactaactattg gctgctgg-3′ (fru-40) and fru-26-rev; (G) an additional set of primers that amplify the male P1 product was used in the case of the Cha^{M5}/fru^{w12} mutant combination: forward primer 5′-gcattacgcggccttggact-3′ (fru-28) and reverse primer fru-26-rev. PCR products were size fractionated on agarose gels and analyzed on Southern blots to confirm that they were *bona fide fru* products. Note that each of the primer sets used amplifies a product that contains at least one splice junction so that products generated from contaminating genomic DNA, if any, are distinguishable from those generated from *fru* transcripts. The identity of the PCR products detected in these experiments was confirmed in all cases by probing these Southern blots with end-labeled oligonucleotide probes homologous to *fru* mRNA sequences expected to be amplified by each primer pair or with probes that span at least part of the expected *fru* products. These oligo probes used did not overlap the primer sequences used for amplification.

In situ hybridization to fru mutant animals: In situ hybridizations to *fru* mutant animals were carried out as described in Goodwin *et al.* (2000) on 20-μm horizontal cryostat sections. Single-stranded riboprobes were synthesized from fragments of *fru* cDNAs subcloned into Bluescript pSK(+). The labeling reaction used T3 or T7 polymerase with digoxygenin-coupled nucleotides according to manufacturer's instructions (DIG RNA labeling kit; Boehringer Mannheim, Indianapolis). Yields were estimated by dot blot of serially diluted labeled probes

by comparison with prelabeled RNA standards (Boehringer Mannheim). The P1 promoter probe (Probe S; Ryner *et al.* 1996) included nucleotides 160,236–159,918 of ACC no. AE003722, plus nucleotides 1–261 of ACC no. U27492. The common coding region probe (Probe C; Ryner *et al.* 1996) consisted of nucleotides 2785–3612 in ACC no. U72492.

Behavioral analysis of *fru* mutants: Adult *fru* mutant males were tested for a variety of courtship and noncourtship behaviors. To test for sterility, males were collected at eclosion and stored either individually or as a group of 8–10 males per vial. After aging for 3–4 days, single males were placed with two to three wild-type virgin females in food vials and the presence or absence of the progeny was scored 7 days later (Gailey and Hall 1989; Gailey *et al.* 1991; Villella *et al.* 1997). Vials in which the males had died were not counted.

Males to be tested for their courtship behavior were collected just after eclosion and aged individually for 6–10 days. The test male was placed in a small observation chamber with either another male of the same genotype or a virgin Canton-S female. The pair was then video recorded for 5–8 min (*cf.* Villella *et al.* 1997). The percentage of time that a male spends courting [courtship index (CI)] another male (CI $m \rightarrow m$) or female fly (CI $m \rightarrow f$) during the observation period was determined by viewing the video tapes and logging the behaviors with a digital event recorder, leading to elementary computations of accumulated-time-logged/total-observation-time for a given playback (Villella *et al.* 1997). When the male is paired with another male, the CI is calculated only for the first male to initiate courtship toward the other male. When the male is paired with a female, the CI represents the time that the male courts the female. The wing extension index (WEI; Villella *et al.* 1997), which is the amount of time that the male extended his wing during the observation period, was also calculated. In addition, courtship song was simultaneously recorded onto the videotapes, and then a subset of the $m \rightarrow f$ recording was analyzed for various song parameters using LifeSong (Villella *et al.* 1997).

Observations of male courtship behavior in all-male groups were used to assess the degree that mutant males courted each other. Males were collected at eclosion and aged individually for 5–6 days. After grouping eight males of the same genotype together in a food vial for 3–4 days, an observation of multimale courtship interactions for each genotype was made in the late afternoon or early evening. Animals were housed and observations were made under standard conditions of 25° and 70% humidity. A courtship chain was defined as an episode during which three or more of the eight males courted one another. The percentage of time that three or more males courted in such group interactions during a 10-min observation period was logged using a timer and computed as the chaining index (ChI; Villella *et al.* 1997).

General activity measurements: To measure short-term locomotor activity, males were collected at eclosion and aged individually for 7–9 days (Kulkarni and Hall 1987). Single males were then placed in a small plexiglass chamber (1 cm diameter × 6 mm height) in which a filter paper with a single line dividing the chamber into two equal halves was placed at the bottom. After a 2- to 3-min accommodation period (which started just after introducing males into the chambers) the number of times each male crossed the center line in a 3-min observation period was counted with a hand-held counter. These observations were done in the late afternoon, usually 2–4 hr before the lights go off.

Statistics: CIs and ChIs were subjected to arcsine square root transformations (*cf.* Villella *et al.* 1997) and then the studentized residuals were tested for normal distribution approximations (Sokal and Rohlf 1995). Short-term activity and courtship song (interpulse intervals) data were not transformed. One-way ANOVAs, followed by subsequent planned pairwise comparisons, were performed on all behavioral and song data (see table legends for further statistical details).

Lethal phase and anatomical analysis of *fru* lethal mutants: *Viability and lethal phase determination:* To determine the lethal phase for various *fru* mutant genotypes, pupae and larvae were collected from the appropriate cross, staged, and allowed to develop. For genotypes where animals reached pharate adult stages, the operculum was removed from pupae and they were allowed to emerge, or they were dissected out of the pupal case. The adults obtained were kept in small groups on agar plates supplemented with yeast**.** In some cases, their behavior was videotaped.

General anatomical and neuroanatomical analysis: fru mutant animals were subjected to several types of anatomical analysis to characterize morphological phenotypes. For external cuticle preparations, adult or late stage pharate adult animals were fixed in alcohol, macerated in hot 10% KOH, dehydrated through an alcohol series and xylene, and mounted between two coverslips in Permount (Szabad 1978). To examine the motorneuronal innervation of abdominal and genital muscles, adult abdomens were dissected, fixed in 4% paraformaldehyde in PBS, and prepared for immunohistochemistry. Nerve terminals were labeled using an anti-synaptotagmin antibody (1:1000 in PBS, 0.1% Triton-X and 2–10% normal goat serum; Littleton *et al.* 1993) to label synapses by the protocol described in Finley *et al.* (1997). Since all *fru* lethal genotypes survived past the third instar larval stage, we examined the division pattern of a set of sex-specific abdominal neuroblasts by 5′ bromodeoxyuridine (BrDU; Sigma, St. Louis) incorporation. Dissected larval and early pupal CNSs were incubated in BrDU for 4 hr and then processed for immunohistochemistry according to techniques in Taylor and Truman (1992).

All immunohistochemical analyses used biotinylated secondary antibodies and ABC reagents (Vector kit; Vector Laboratories, Burlingame, CA). The color reaction used diaminobenzidine for visualization according to standard techniques (Taylor and Truman 1992; Taylor and Knittel 1995).

Muscle of Lawrence analysis of fru mutants: Procedures used for the characterization of the muscle of Lawrence (MOL) are detailed elsewhere (Gailey *et al.* 1991). In brief, dorsal abdominal cuticles were dissected out, fixed, and cleared in methyl salicylate. Musculature was then visualized by birefringence in polarized light. Individual MOL phenotypes were established by a ranking system outlined in Villella *et al.* (1997); see also Table 8 legend.

RESULTS

Genetic and molecular analysis of the new *fru* mutants

Isolation of new *fru* mutants: To generate new, more severe *fru* mutations, we mutagenized the fru^2 and fru^4 mutations (Gailey and Hall 1989; Castrillon *et al.* 1993). Both of these *fru* mutants are due to *P*-element inserts: the fru^2 *P* element is located between the 5′ exons of the P3 and P4 promoters and fru^4 is between the 5′ exons of the P2 and P3 promoters (Figure 1B; Ito *et al.* 1996; Ryner *et al.* 1996; Goodwin *et al.* 2000). The fru^2 *P* element was mutagenized by X rays and the fru^4 *P* element was mobilized using standard techniques to generate imprecise excisions (see materials and methods). From these mutageneses, we recovered eight *fru* mutations.

Cytological and molecular characterization of new *fru*

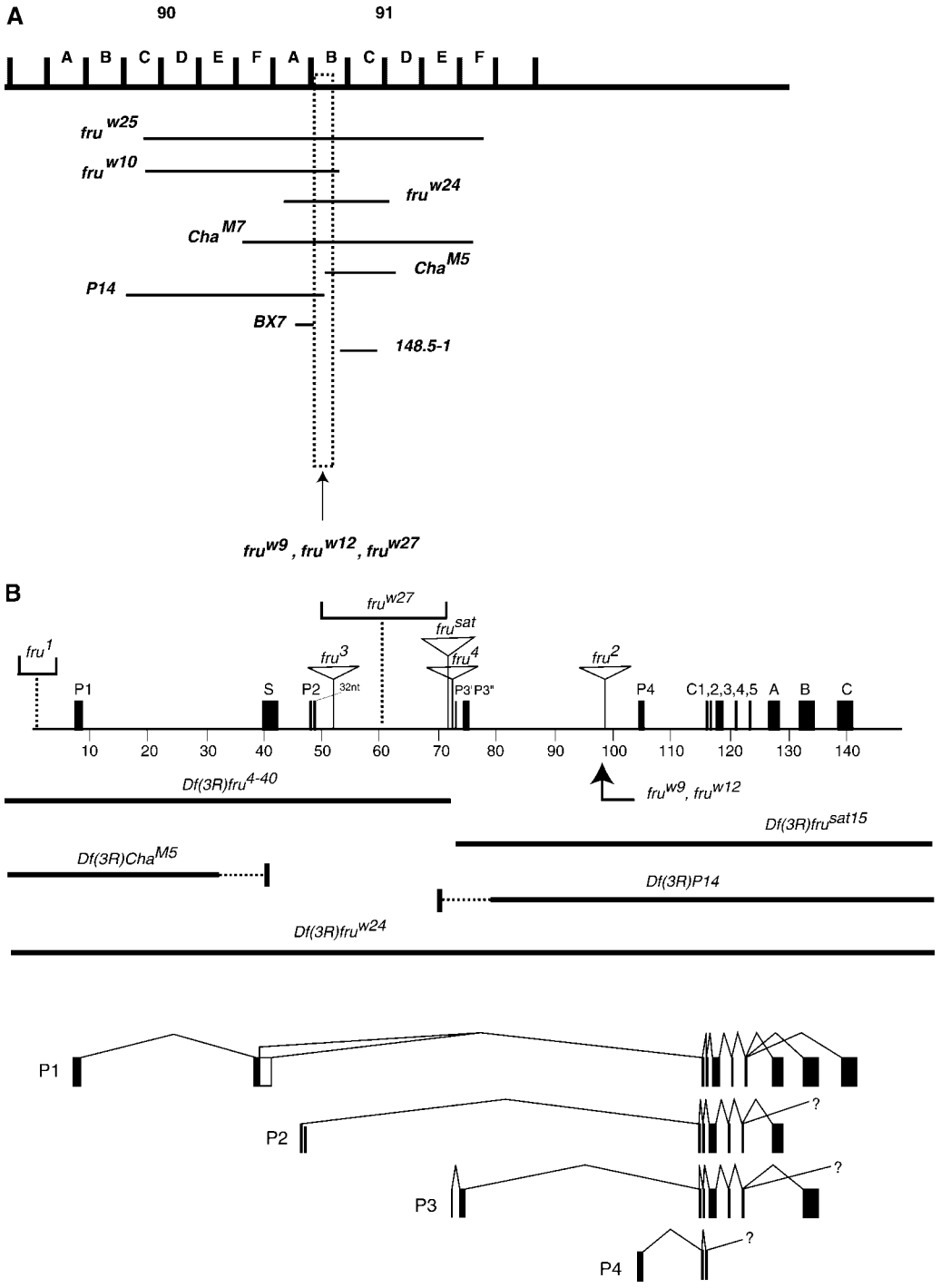

FIGURE 1.—Genetic and molecular map of the *fru* locus. (A) The cytological map of the 90–91 region (right arm of the third chromosome). For deficiencies, the deleted regions are indicated by thick solid lines (see Table 1 for details of the mutations). The locations of the chromosomal breaks in fru^{w9}, fru^{w12}, and fru^{w27} alleles are denoted by the arrow. (B) The molecular map of the *fru* locus. The insertion sites of the fru^{2}, fru^{3}, fru^{4}, and fru^{sat} *P* elements and the breakpoints of the fru^{w9}, fru^{w12}, and fru^{w27} chromosomal aberration mutations are shown at a higher level of resolution. The ends of relevant deficiencies are shown, with the thick solid lines demarcating missing DNA and the dashed lines representing the restriction fragments to which the breaks were mapped. The positions of the four promoters (P1–P4) are mapped to their relevant restriction fragments of the *fru* genomic DNA (adapted from RYNER *et al.* 1996). Each alternative 3′ end encodes a pair of Zn-f domains and is labeled A, B, or C according to its distal-to-proximal location in the gene. The P1 transcripts are sex-specifically spliced by the action of the TRA and TRA2 proteins interacting with 13-nucleotide (nt) repeats, resulting in sex-specific transcripts that produce proteins differing at their amino termini. By Northern analysis, three P1 transcript classes have been detected in male and female heads that correspond to usage of each of the alternative 3′ ends (GOODWIN *et al.* 2000). The full transcript complexity is not known for transcripts from the other promoters and so the 3′ ends for P2, P3, and P4 are not included. Note that the orientation of the molecular map is reversed with respect to the cytological map.

mutants: Cytological examination of salivary chromosomes showed that six of the eight new fru^{-} mutations had visible aberrations (Table 1). The $Df(3R)fru^{w10}$, $Df(3R)fru^{w24}$, and $Df(3R)fru^{w25}$ alleles are deficiencies uncovering the 91B region of the third chromosome. $In(3LR)fru^{w12}$ is an inversion and $T(3;Y)fru^{w9}$ is a translocation, each with a chromosomal break at 91B**,** the location of *fru*. $T(3R;het)fru^{w12}$ is an inversion-cum-translocation also broken in 91B. Putting the findings that three of the fru^{2}-derived mutations are inversions or translocations with one breakpoint at the cytological location of *fru* together with the fact that these rearrangements arose simultaneously with the inactivation of the wild-type *white* gene in the fru^{2} *P* element suggests that these three rearrangements are broken in the fru^{2} *P* element.

Southern analysis was used to examine whether these

TABLE 1

Cytology of *fru* mutations

Aberration/mutation	Cytology	Origin	Reference
Deficiencies			
Df(3R)fru^{w24}, gl$^-$ fru$^-$	91A1; 91D2–3	*fru^2* X ray	Ryner *et al.* (1996)
Df(3R)Cha^{M5}, sr$^+$ gl$^+$ fru$^-$ Cha$^-$	91B; 91D	X ray	Gailey and Hall (1989)
Df(3R) fru^{w25}, gl$^-$ fru$^-$	90C4–5; 91F10–11	*fru^2* X ray	This article
Df(3R) fru^{w10}, gl$^-$ fru$^-$	90C3–6; 91B4–11	*fru^2* X ray	This article
Df(3R)Cha^{M7}, sr$^+$ gl$^-$ fru $^-$ Cha$^-$	90F; 91F	γ ray	Gailey and Hall (1989)
Df(3R)148.5-1, sr$^+$ gl$^+$ fru$^+$ Cha$^-$	91B3; 91D1	γ ray	Gailey and Hall (1989)
Df(3R)BX5, sr$^+$ gl$^+$	91B1–2; 91D1–2	X ray	Moses *et al.* (1989)
Df(3R)BX7	Normal	X ray	Moses *et al.* (1989)
Df(3R)P14, sr$^-$ gl$^-$ fru$^-$	90C2–D1; 91B1–2	X ray	Gailey and Hall (1989)
Df(3R)fru$^{4-40}$, fru$^-$	Normal	*fru^4* mobilization	This article
Df(3R)fru^{sat15}, gl$^+$fru$^-$	Normal	*fru^{sat}* mobilization	Ito *et al.* (1996)
Inversions and translocations			
In(3R + 3LR)fru^{w27}	In(3R) 88C; 91B1 −2 + In (3LR) 68A–B; 92E	*fru^2* X ray	Ryner *et al.* (1996)
In(3R)fru^1	90C; 91B	X ray	Gailey and Hall (1989)
T(3:Y)fru^{w9}	91B1–2; het[a]	*fru^2* X ray	This article
In(2LR) + T(3,het)fru^{w12}	In(3LR)65C–D; 91B1-2; T(3,het) 91B1-2, het[b]	*fru^2* X ray	Ryner *et al.* (1996)
fru^{w13}	Normal	*fru^2* X ray	This article
P-element alleles			
fru^2	*P*{(*w, ry*) A}	—	Levis *et al.* (1985)
fru^3	*P*{*lacZ, ry$^+$*}	—	Castrillon *et al.* (1993)
fru^4	*P*{*lacZ, ry$^+$*}	—	Castrillon *et al.* (1993)
fru^{sat}	*P*{*lwB*}	—	Ito *et al.* (1996)

A list of the mutations used in this study with their chromosomal cytology, the source of the mutation, and their most recent reference is shown.

[a] het refers to a breakpoint in salivary gland heterochromatin.

[b] Order is 100-91B-65CD-cent-91B-het; 61-65CD-het.

three rearrangements were broken in the *fru^2 P* element. These experiments showed that *In(3LR)fru^{w12}* and *In(3R)fru^{w27}* had lesions within a 4-kb *Hin*dIII fragment at the 5′ end of the *white$^+$* gene in the *P* element. *T(3;het) fru^{w9}* has a lesion located within a 3-kb *Bam*HI fragment, partially overlapping the proximal end of the 4-kb *Hin*dIII fragment. These data are thus consistent with the idea that the *fru* breakpoints associated with these three rearrangements are within the *fru^2 P* element. Thus these three rearrangements should separate the P1-3 *fru* promoters, but not the P4 promoter, from the common *fru* coding sequences.

We also molecularly mapped the tiny deletions associated with the *fru* mutations *Df(3R)fru$^{4-40}$* and *Df(3R)fru^{sat15}*. *Df(3R)fru$^{4-40}$* was found to extend distally from within the *fru^4 P* element for at least 70 kb (see materials and methods). *Df(3R)fru^{sat15}*, which is derived from the excision of a *P* element inserted into the *fru* gene (Ito *et al.* 1996), extends centromere-proximal from within the *fru^{sat}* insert and removes the entire common *fru* coding region as well as several adjacent genes (Figure 1; see materials and methods).

Transcript analysis of *fru* mutants: Since the new *fru* mutants described above are either deletions of portions of the *fru* locus or inversions or translocations that separate some *fru* promoters from *fru* coding sequences, these mutants are expected to lack various subsets of *fru* transcripts. They can thus be used to dissect the functions of various *fru* transcript classes. Rather than rely on results of the above cytological and molecular characterizations to infer which transcripts are produced by these mutant chromosomes, we used reverse transcription (RT)-PCR to directly examine the arrays of transcripts produced by various genotypes [*In(3R)fru^{w27}/ Df(3R)fru^{w24}*, *T(3R;het)fru^{w12}/ Df(3R)fru^{w24}*, *T(3R;het)fru^{w12}/ In(3R)fru^{w27}*, *Df(3R)fru^{sat15}/ Df(3R)fru$^{4-40}$*], and *Df(3R)Cha^{M5}/ T(3R;het)fru^{w12}* mutant animals and *Df(3R)fru^{w24}*/+ adults; the latter were used as a positive control]. RNA was extracted from these genotypes and reverse transcribed. Following the RT reactions aliquots of each reaction were amplified with various primer pairs (see materials and methods) to determine which transcript classes were produced in each genotype.

We first determined whether any *fru* transcripts were produced in these genotypes by using primers that amplified a sequence from the protein-coding region com-

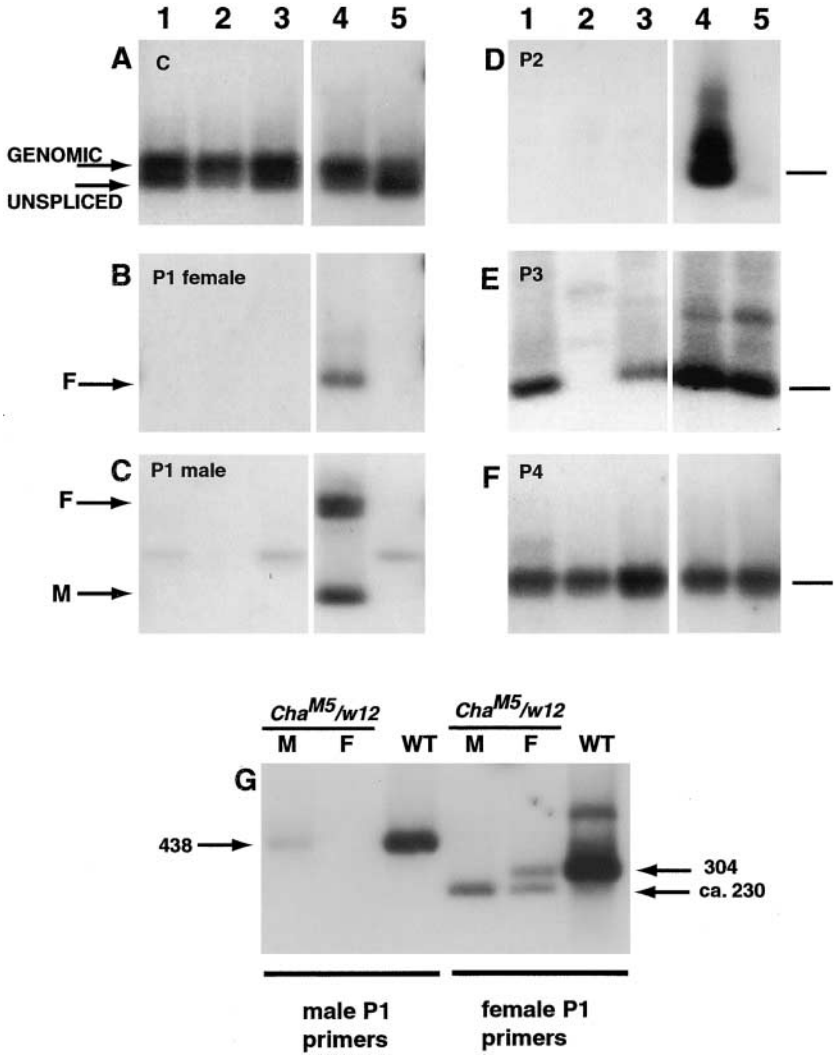

FIGURE 2.—RT-PCR analysis of *fru* mutants for the detection of sex-specific and sex-nonspecific transcripts. In A–G, lanes are as follows: lane 1, fru^{w27}/fru^{w24}; lane 2, fru^{w12}/fru^{w24}; lane 3, fru^{12}/fru^{w27}; lane 4, $fru^{w24}/+$; lane 5, $fur^{4\text{-}40}/fru^{sat15}$. Arrows point to the *fru*-specific bands. F, female-specific product; M, male-specific product. (A) The 317-nt RT-PCR product amplified from the *fru* common protein-coding region, which amplifies transcripts from all of the *fru* promoters (primers fru-25 and fru-26-rev). The slightly larger product detected is due to contamination of genomic DNA, which contains a 72-nt intron. Transcripts containing *fru*-coding sequences were detected in all fru^{w24}/fru^{w27}, fru^{w12}/fru^{w24}, fru^{w12}/fru^{w27}, and $fru^{4\text{-}40}/fru^{sat15}$ mutants. Midstage pupae were used for fru^{w24}/fru^{w27}, fru^{w12}/fru^{w24}, and fru^{w12}/fru^{w27} and adults were collected for the control. (B) The 305-nt RT-PCR product of female-specific P1 transcripts (primers fru-2 and fru-7-rev). Transcripts were detected in control $fru^{w24}/+$ but no P1 transcripts were detected in any of the mutants. (C) The 1931- and 341-nt RT-PCR products amplified from P1 transcripts using primers (fru-31 and fru-26-rev) to amplify a sequence, which includes the male-specific splice to the common coding region. In the control $fru^{w24}/+$ lane, both male-specific (lower band) and female-specific transcripts (upper band) are detected with these primers. No wild-type P1 transcripts were detected in any of the mutants. Bands in the fru^{w27}/fru^{w24}, fru^{w12}/fru^{w27}, and $fru^{4\text{-}40}/fru^{sat15}$ lanes are not the expected size and are likely to be artifacts of the PCR. (D) The 379-nt RT-PCR product amplified from P2 transcripts (primers fru-29 and fru-55-rev-Xho). P2 transcripts were only detected in the control $fru^{w24}/+$ lane. A faint band was present in the $fru^{sat15}/fru^{4\text{-}40}$ lane but is smaller than the expected size and so is likely an artifact. (E) The 255-nt RT-PCR product amplified from P3 transcripts (primers fru-12 and fru-7-rev). P3 transcripts were not detected in fru^{w12}/fru^{w24} animals. (F) The 360-nt RT-PCR product amplified from P4 transcripts (primers fru-40 and fru-26-rev). The fru^{w24}/fru^{w27}, fru^{w12}/fru^{w24}, fru^{w12}/fru^{w27}, and $fru^{4\text{-}40}/fru^{sat15}$ mutants make P4 *fru* transcripts. (G) The 438-nt male P1 and 304-nt female P1 RT-PCR products amplified from total RNA of *ChaM5/W12* adult flies (male primers fru-28 and fru-26-rev, female primers fru-2 and fru-7-rev). An ~230-nt product is also detected with the female primers and may represent use of a 5′ splice site 73 nt upstream of the female-specific 5′ splice site. Note this product is detected in both males and females.

mon to all known transcript classes. This experiment detected *fru* transcripts in all five mutant genotypes (Figure 2A). These findings are as expected, since the region between P4 and the common coding region is normal on one of the chromosomes in each of these genotypes.

Whether the sex-specifically spliced P1 transcripts were produced in these mutants was tested with two sets of primers. One pair amplified the female product (Figure 2B) and another pair amplified both the male and female-specific products (Figure 2C), since the sequence of the P1 transcripts in males overlaps that in females. These primers amplified the expected products from control animals (Figure 2, B and C). No P1 transcripts were detected from $In(3R)fru^{w27}/\ Df(3R)fru^{w24}$, $Df(3R)fru^{w24}/\ T(3R;het)fru^{w12}$, $T(3R;het)fru^{w12}/\ In(3R)fru^{w27}$, and $Df(3R)fru^{4\text{-}40}/\ Df(3R)fru^{sat15}$ animals (Figure 2, B and C; data not shown). These findings are consistent with expectations since $In(3R)fru^{w27}$ and $T(3R;het)fru^{w12}$ are broken between P1 and the common coding sequences, $Df(3R)fru^{sat15}$ deletes the common coding sequences, $Df(3R)fru^{4\text{-}40}$ deletes the P1 promoter, and $Df(3R)fru^{w24}$ deletes the entire *fru* locus. The absence of P1 transcripts in these mutant combinations was confirmed by carrying out two rounds of nested PCR with primers that amplified the female product (data not shown).

Surprisingly, P1 transcripts were detected in the $Df(3R)Cha^{M5}/\ T(3R;het)fru^{w12}$ mutant combination (Figure 2G) with both sets of primers. As shown in other mutant combinations, $T(3R;het)fru^{w12}$ does not produce P1 transcripts spliced to the common coding region and thus these transcripts must come from the $Df(3R)Cha^{M5}$ chromosome. $Df(3R)Cha^{M5}$ is a large deletion beginning

between the P1 and P2 promoters and extending distally far beyond the P1 promoter. Thus the RT-PCR product detected cannot be a *fru* transcript initiated from the P1 promoter (Figure 1B). However, the end of the *Df(3R)Cha*^{M5} deletion that is within *fru* is located in the same large genomic restriction fragment that contains the 5′ primer sequences used for the RT-PCR detection of P1 transcripts (Figure 1B). The results showing that RT-PCR products were detected with both the male and female primers indicate that the sequences corresponding to these primers are not deleted. Note that the authenticity of all PCR products was confirmed by Southern analysis using probes that did not contain the primer sequences (see MATERIALS AND METHODS). Therefore the most likely explanation for the "P1" transcripts detected in *Df(3R)Cha*^{M5}*/T(3;het)fru*^{w12} is that these transcripts are being produced by an ectopic promoter that was juxtaposed to *fru* by the *Df(3R)Cha*^{M5} deletion. If this is the case, these P1 transcripts may not be being expressed in the cells in which the P1 *fru* promoter is normally active. Indeed, antibodies specific to the *fru* male-specific proteins expressed from the P1 promoter fail to detect these proteins in whole mounts of central nervous systems of *Df(3R)Cha*^{M5}*/Df(3R)P14* at the pupal stages when these proteins are expressed in wild type (LEE *et al.* 2000).

In addition to the sex-specific P1 products detected in *Df(3R)Cha*^{M5}*/T(3R;het)fru*^{w12}, a sex-nonspecific product, slightly smaller than the wild-type product, is detected in both sexes with the female-specific primers. There is a 5′ splice site consensus sequence upstream of the female 5′ splice site that would give a product of this size if it were used. Sex-nonspecific products using this splice site have not been detected in wild type or in any of the other mutants and thus are specific to the *Df(3R)Cha*^{M5} deficiency. There are two possible explanations for why this site might be used in *Df(3R)Cha*^{M5}-derived transcripts. Because this shorter product is produced in both sexes, it may be the result of ectopic promoter-driven expression in tissues where this cryptic splice site can be utilized. Alternatively, the *Df(3R)Cha*^{M5} deletion may remove upstream sequences that affect the use of this splice site. The latter explanation seems less likely because the primary P1 transcript from *Df(3R)Cha*^{M5} contains sequences (the male-specific PCR primer) upstream of the male 5′ splice site, which is >1.6 kb away from the regulatory sequences that are known to affect the regulation of the female-specific splice site in wild type (HEINRICHS *et al.* 1998). These results indicate that a novel *fru* transcript is produced from the *Df(3R)Cha*^{M5} deficiency chromosome; whether this transcript is expressed in the proper cells to supply *fru* function is unknown, but phenotypically there is no evidence for P1 function (see below).

On the basis of the location and nature of the *fru* rearrangements in these genotypes, transcripts initiated at the P2 promoter and spliced to the common coding region are not expected to be present except in the case of the *Df(3R)Cha*^{M5}*/ T(3R;het)fru*^{w12} genotype. As predicted, no P2 transcripts were detected in any of the *fru* mutant genotypes tested except for *Df(3R)Cha*^{M5}*/ T(3R;het)fru*^{w12} (Figure 2D, data not shown). In the latter genotype the *Df(3R)Cha*^{M5} allele is the likely source of the P2 RT-PCR products since it does not delete the P2 promoter (Figure 1B); and the *T(3;het)fru*^{w12} allele alone, as shown in the *Df(3R)fru*^{w24}*/ T(3R;het)fru*^{w12} lane (Figure 2D), does not produce a P2 product spliced to the common coding region.

With respect to the P3 promoter, the locations of the breakpoints in these *fru* alleles predict that only the *Df(3R)Cha*^{M5} and *Df(3R)fru*$^{4\text{-}40}$ chromosomes should produce P3 transcripts spliced to the common coding region. These expectations are fulfilled in all but one case (Figure 2E). Unexpectedly, in all genotypes involving the *In(3R)fru*^{w27} allele, RT-PCR revealed P3 transcripts. That these products are real is indicated by the facts that they are of the correct size and that they contain the expected *fru* mRNA sequences between the PCR primer pairs (see MATERIALS AND METHODS). These findings suggest that *In(3R)fru*^{w27} is more complex than indicated by its cytological and molecular characterization. The *In(3R)fru*^{w27} chromosome has one inversion with a break in *fru* and a second inversion that does not involve *fru*. Southern analysis indicated that there was a lesion within the *white* gene of the *fru*^{2}-associated *P* element on which *In(3R)fru*^{w27} was induced, and we had inferred that this was the breakpoint of the *In(3R)fru*^{w27}-associated inversion. However, the RT-PCR findings that P3 but not P2 transcripts are produced from the *In(3R)fru*^{w27} chromosome suggest that there is a breakpoint in the region between the P2 and P3 promoters as shown in Figure 1B.

In the case of the P4 promoter, all the *fru* alleles analyzed, except *Df(3R)fru*^{sat15} and *Df(3R)fru*^{w24} (both of which delete P4), are expected to produce P4-derived transcripts spliced to the common coding region. The results (Figure 2F) are in accord with these expectations.

In summary, the above data show that different *fru* mutants are missing different subsets of *fru* transcripts (Table 2). As described above, our results are consistent with the idea that *Df(3R)Cha*^{M5} affects only P1 function. Two mutations, *Df(3R)fru*$^{4\text{-}40}$ and *In(3R)fru*^{w27}, lack both P1- and P2-derived transcripts. One mutation, *T(3;het)-fru*^{w12}, lacks P1-, P2-, and P3-derived transcripts, and we infer that *T(3;Y)fru*^{w9} does also on the basis of the location of its breakpoint (see above). Finally, *Df(3R)fru*^{sat15}, *Df(3R)fru*^{w24}, and the previously characterized deletions *Df(3R)P14* and *Df(3R)Cha*^{M7} lack functional products from all *fru* promoters but also delete neighboring genes. By using different combinations of these *fru* alleles, we have begun to associate specific *fru* mutant phenotypes with the loss of different *fru* transcript classes.

TABLE 2

Summary of transcript classes expressed by *fru* alleles

fru allele	Transcript class P1	P2	P3	P4
$fru^{1\,a}$	+	+	+	+
$fru^{2\,a}$	(+)	(+)	(+)	+
$fru^{3\,a}$	(+)	(+)	+	+
$fru^{4\,a}$	(+)	(+)	+	+
$Cha^{M5\,b}$	(+)[d]	+	+	+
$fru^{4\text{-}40\,b}$	−	−	+	+
$fru^{w27\,b,e}$	−	−	+	+
$fru^{w12\,b}$	−	−	−	+
$fru^{w9\,c}$	−	−	−	+
$fru^{w13\,c}$	−	−	−	+
$fru^{sat15\,b}$	−	−	−	−
$fru^{w24\,b}$	−	−	−	−
$P14^{c}$	−	−	−	−

The + indicates those transcripts that have been detected by Northern analysis in *fru* mutant heads (Goodwin *et al.* 2000) or by RT-PCR analysis in this article. The transcript levels indicated by a (+) are those where levels are reduced, since these were detectable by RT-PCR of poly(A)+-selected RNA but not by standard RT-PCR (Goodwin *et al.* 2000). For those *fru* alleles in which *fru* transcripts were not detected by RT-PCR or where their absence is inferred from genetic analysis, the absence of transcripts is denoted by a minus sign.

[a] Based on Northern and RT-PCR analyses (L. C. Ryner, S. F. Goodwin, T. Carlo, M. Foss, J. C. Hall, B. J. Taylor and B. S. Baker, unpublished results).

[b] Based on RT-PCR analyses (this study).

[c] Inferred from genetic analysis of cytology and mutant phenotype (this study; Gailey *et al.* 1991).

[d] These are likely transcripts produced by an ectopic promoter juxtaposed to *fru* by the Cha^{M5} deletion.

[e] Genetic analysis suggests that fru^{w27} lacks *fru*'s vital function and thus likely does not produce functional P3 (or perhaps P4) products.

Phenotypic analysis of new *fru* mutants

Origin of *fru*'s vital function: To determine which *fru* promoters encode its vital function(s) we examined the viability of various heteroallelic *fru* mutant combinations that lacked particular subsets of *fru* transcripts (Table 3).

The finding that $Df(3R)Cha^{M5}/Df(3R)fru^{sat15}$ individuals, which have little or no P1 function and express the products of the other *fru* promoters, have normal viability (Table 3B) indicates that the products of the P1 *fru* promoter are not needed for viability. This conclusion is also supported by the findings that $Df(3R)Cha^{M5}$ is also viable over *Df(3R)P14* (Gailey *et al.* 1991). *Df(3R)P14* is like $Df(3R)fru^{sat15}$ in that it is a deletion that has one end in the middle of *fru* and extends proximally, deleting all *fru* coding sequences as well as neighboring genes.

The RT-PCR experiments described above suggest there are two aberrations, $Df(3R)fru^{4\text{-}40}$ and $In(3R)fru^{w27}$, which lack P1 and P2 function, but have P3 and P4 function. However, these two aberrations give completely discordant results when used in complementation tests with other *fru* alleles that lack *fru*'s vital function. Thus $Df(3R)fru^{4\text{-}40}$ over $Df(3R)fru^{sat15}$ or *Df(3R)P14* is fully viable (Table 3C), whereas $In(3R)fru^{w27}$ over $Df(3R)fru^{sat15}$, $Df(3R)Cha^{M7}$, $Df(3R)fru^{w24}$, $T(3;het)fru^{w12}$, or $T(3;Y)fru^{w9}$ is lethal (Table 3D). As explained in the discussion we believe that this disparity is due to $In(3R)fru^{w27}$ being defective in more than just *fru*'s P1 and P2 functions and that the $Df(3R)fru^{4\text{-}40}$ results are reflective of the phenotype resulting from the loss of both P1 and P2 functions. This would indicate that P2-derived products, like those derived from P1, are not needed for viability.

With respect to P3, our analysis suggests that both $T(3;het)fru^{w12}$ and $T(3;Y)fru^{w9}$ do not produce P3-, P1-, or P2-derived products. Both $T(3;het)fru^{w12}$ and $T(3;Y)fru^{w9}$ are lethal over the null *fru* mutants $Df(3R)fru^{sat15}$, $Df(3R)fru^{w24}$, and $Df(3R)Cha^{M7}$, as well as each other (Table 3D). These results indicate that the products of P3 are essential for viability.

We do not have a genotype with which we can assess the viability effects of losing P4 but not P3 function. While we examined the viability effects of genotypes that are complete nulls for *fru* (Table 3E), and these are invariably lethal as expected, these deficiency combinations are also deleted for genes flanking *fru*. Nevertheless the phenotypic characterization of the lethal phenotypes in these deficiency combinations (see below) is useful as it places an upper boundary on the severity of the phenotype that can be expected from a null *fru* genotype.

Male sterility of the *fru* mutants: Previous studies established that males carrying many *fru* mutants and mutant combinations are sterile and that this sterility is behavioral in origin: Such males frequently fail to copulate (Gailey and Hall 1989; Gailey *et al.* 1991; Villella *et al.* 1997). In addition, it was recently found by examining *fru* mutant combinations that do sometimes mate that *fru* function is also required during mating for successful insemination (Lee *et al.* 2001). To gain insight into which *fru* products are required for male fertility we assayed the effects of the new *fru* alleles on fertility.

That $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{w24}$, $In(3R)fru^{w27}$, $Df(3R)fru^{sat15}$, and $Df(3R)fru^{4\text{-}40}$ all abolish male fertility is shown by the complete sterility of all combinations of these mutants tested with each other (Table 4D). Since all the genotypes in Table 4D, lack the functions encoded by the P1 and P2 *fru* promoters, these results indicate that some product(s) from these promoters are required for male fertility. To examine whether the products of the P1 promoter were required for male fertility we examined the effects of *trans*-heterozygotes between $Df(3R)Cha^{M5}$ and $T(3;Y\,fru^{w9}$, $T(3;het)fru^{w12}$, $In(3R)fru^{w27}$, $Df(3R)fru^{4\text{-}40}$, or $Df(3R)fru^{sat15}$. With one exception, all combinations were completely sterile (Table 4C). The exception was that in one round of tests 3/65 $Df(3R)Cha^{M5}/In(3R)fru^{w27}$ males were fertile, whereas sev-

TABLE 3

Viability of *fru* mutants

Genotype	No. of viable adults	Genotype	No. of viable adults
A. fru^{+} control genotypes			
$fru^{sat15}/BX5$	49 (148)		
B. *fru* genotypes with P2, P3, and P4 functions, but little or no P1 functions[a,b]			
fru^{sat15}/Cha^{M5}	304 (904)	$fru^{4\text{-}40}/Cha^{M5}$	0 (292)
fru^{w24}/Cha^{M5}	0 (702)	Cha^{M5}/P14	208 (823)
C. *fru* genotypes with just P3 and P4 functions			
fru^{w27}/fru^{sat15}	27 (594)	$fru^{4\text{-}40}/P14$	422 (1103)
$fru^{4\text{-}40}/fru^{sat15}$	427 (1131)	$fru^{4\text{-}40}/fru^{w24}$	0 (895)
D. *fru* genotypes with just P4 functions[c]			
fru^{w9}/fru^{w27}	1 (960)	fru^{w24}/fru^{w27}	0 (187)[d]
fru^{w9}/fru^{w24}	0 (266)	fru^{w12}/fru^{w24}	0 (560)[d]
fru^{w9}/fru^{w12}	0 (3057)	fru^{w12}/Cha^{M7}	0 (147)
fru^{w9}/fru^{sat15}	0 (463)	fru^{w12}/fru^{sat15}	13 (817)
fru^{w9}/Cha^{M7}	0 (186)	fru^{w27}/Cha^{M7}	0 (316)
$w+; fru^{w12}/fru^{w27}$	19 (1103)		
$w; fru^{w12}/fru^{w27}$	14 (1129)		
E. *fru* genotypes expressing no *fru* transcripts			
$fru^{sat15}/P14$	0 (737)	fru^{w24}/Cha^{M7}	0 (218)
fru^{sat15}/fru^{sat15}	0 (350)	$fru^{4\text{-}40}/Cha^{M7}$	0 (291)
fru^{sat15}/Cha^{M7}	0 (396)	$fru^{w24}/P14$	0 (324)
fru^{sat15}/fru^{w24}	0 (672)		

The number of viable *fru* mutant adults found for each genotype is shown, with the total number of animals analyzed in parentheses. The following genotypes were fully viable: $fru^{sat15}/BX5$, fru^{sat1}/Cha^{M5}, $fru^{4\text{-}40}/P14$, and $fru^{4\text{-}40}/fru^{sat15}$, since the proportion of mutant animals in each case was about one-third (range from 32–42%) of the total number of adults, as would be expected for normal Mendelian segregation. The following genotypes had only rare adult escapers: fru^{w9}/fru^{w27} (0.1%), fru^{w12}/fru^{w27} (w^{-}, 1% and w^{+}, 2%), fru^{w27}/fru^{sat15} (5%), and fru^{w12}/fru^{sat15} (2%). In other genotypes, no escapers were found but viable adults could be dissected from the pupal case (see Table 8). Only 10% of the expected numbers of fru^{2}/fru^{W24} flies were found compared to the Balancer siblings (107 fru^{2}/fru^{w24} out of 1114 total flies scored).

[a] While P1 transcripts were detected by RT-PCR, these likely supply little or no *fru* P1 function (see text).

[b] Based on data from Goodwin *et al.* (2000) and this article.

[c] While P3 transcripts are detected by RT-PCR in fru^{w27}, these likely supply little or no *fru* P3 function (see text).

[d] Data from Ryner *et al.* (1996).

eral subsequent rounds of tests found 0/36 fertile. With the exception of these 3 males all the results in Table 4, C and D, are consistent with the hypothesis that functional products of the P1 *fru* promoter are essential for male fertility.

We also examined the effects on male fertility of the new *fru* mutations in combination with the hypomorphic alleles fru^{1}, fru^{2}, fru^{3}, and fru^{4} (Table 4E). *The* fru^{2}, fru^{3}, and fru^{4} alleles are due to *P*-element insertions in *fru* and have reduced levels of either P1- and P2-derived transcripts (fru^{3} and fru^{4}) or reduced levels of P1-, P2-, and P3-derived transcripts (fru^{2}). P4 transcripts are not affected by these mutations (Goodwin *et al.* 2000). fru^{1} is an inversion that removes sequences upstream of the P1 promoter and results in an altered pattern of expression of P1 in the CNS (Goodwin *et al.* 2000). The fru^{3} and fru^{4} alleles show more severe behavioral defects than fru^{1} or fru^{2} (Villella *et al.* 1997). Consistent with the latter observation $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{w24}$, $In(3R)fru^{w27}$, $Df(3R)fru^{4\text{-}40}$, and $Df(3R)fru^{sat15}$ are all completely sterile over either fru^{3} or fru^{4}, whereas a few combinations of these alleles with either fru^{1} or fru^{2} show some male fertility. Two of the latter genotypes show results worthy of note: Most $fru^{1}/Df(3R)fru^{sat15}$ and $fru^{2}/Df(3R)fru^{sat15}$ males are fertile. This is a surprising result, since males with either fru^{1} or fru^{2} over other null deletions of *fru* [*e.g.*, $Df(3R)fru^{w24}$, $Df(3R)Cha^{M7}$, and $Df(3R)P14$] were invariably sterile (Gailey and Hall 1989; Villella *et al.* 1997). This may reflect genetic background effects.

As controls for the above experiments we examined the fertility of the new *fru* alleles when heterozygous with a wild-type chromosome and two deficiencies flanking *fru* (Table 4A). Two flanking deletions, gl^{BX7}, a *glass* allele associated with a 20-kb deletion centromere-proximal to *fru,* and *Df(3R)148.5-1,* a large deletion centromere-distal to *fru*, complemented the sterility phenotype of these new *fru* lesions, showing that there are no

TABLE 4

Percentage of fertile males

Genotype	% fertile	Genotype	% fertile	Genotype	% fertile	Genotype	% fertile
A. fru^+ control genotypes							
fru^+/fru^2	100 (20)	$BX7/fru^2$	100 (20)	$148.5\text{-}1/fru^2$	100 (18)		
fru^+/fru^{w9}	95 (40)	$BX7/fru^{w9}$	100 (18)	$148.5\text{-}1/fru^{w9}$	95 (20)		
fru^+/fru^{w12}	98 (40)	$BX7/fru^{w12}$	100 (11)	$148.5\text{-}1/fru^{w12}$	100 (15)		
fru^+/fru^{w24}	98 (49)	$BX7/fru^{w24}$	Lethal	$148.5\text{-}1/fru^{w24}$	Lethal		
fru^+/fru^{w27}	97 (37)	$BX7/fru^{w27}$	95 (20)	$148.5\text{-}1/fru^{w27}$	81 (53)		
$fru^+/fru^{4\text{-}40}$	100 (10)	$BX7/fru^{4\text{-}40}$	88 (18)	$148.5\text{-}1/fru^{4\text{-}40}$	97 (33)		
fru^+/fru^{sat15}	100 (12)	$BX7/fru^{sat15}$	ND	$148.5\text{-}1/fru^{sat15}$	92 (12)		
B. *fru* genotypes involving hypomorphic *fru* alleles							
fru^1/fru^2	100 (24)	fru^2/fru^2	100 (24)	fru^3/fru^2	23 (30)	fru^4/fru^2	25 (28)
fru^1/fru^{w9}	0 (26)	fru^2/fru^{w9}	7 (56)	fru^3/fru^{w9}	0 (17)	fru^4/fru^{w9}	0 (22)
fru^1/fru^{w12}	0 (20)	fru^2/fru^{w12}	0 (50)	fru^3/fru^{w12}	0 (18)	fru^4/fru^{w12}	0 (27)
fru^1/fru^{w24}	0 (22)	fru^2/fru^{w24}	0 (50)	fru^3/fru^{w24}	0 (23)	fru^4/fru^{w24}	0 (20)
fru^1/fru^{w27}	0 (24)	fru^2fru^{w27}	0 (83)	fru^3/fru^{w27}	0 (20)	fru^4/fru^{w27}	0 (20)
$fru^1/fru^{4\text{-}40}$	0 (19)	$fru^2/fru^{4\text{-}40}$	24 (68)	$fru^3/fru^{4\text{-}40}$	0 (26)	$fru^4/fru^{4\text{-}40}$	0 (21)
fru^1/fru^{sat15}	32 (56)	fru^2/fru^{sat15}	33 (36)	fru^3/fru^{sat15}	0 (19)	fru^4/fru^{sat15}	0 (21)
		fru^2/Cha^{M5}	93 (15)				
C. *fru* genotypes lacking P1 function							
Cha^{M5}/fru^{w9}	0 (30)	Cha^{M5}/fru^{w27}	3 (101)	Cha^{M5}/fru^{sat15}	0 (32)	Cha^{M5}/fru^{w12}	0 (30)
Cha^{M5}/fru^{w2}	Lethal	$Cha^{M5}/fru^{4\text{-}40}$	Lethal				
D. *fru* genotypes with just P3 and P4 functions[a,b]							
$fru^{4\text{-}40}/fru^{w9}$	0 (20)	$fru^{4\text{-}40}/fru^{w27}$	0 (30)	fru^{w12}/fru^{w27}	0 (6)		
$fru^{4\text{-}40}/fru^{w12}$	0 (20)	$fru^{4\text{-}40}/fru^{4\text{-}40}$	Lethal	fru^{w9}/fru^{w27}	0 (1)		
$fru^{4\text{-}40}/fru^{w24}$	Lethal	$fru^{4\text{-}40}/fru^{sat15}$	0 (26)				

The percentage of fertile males is given for each genotype with the total number of males tested in parentheses. Males were aged for 3–5 days in groups and then each male was tested with two to three Canton-S wild-type females in a food vial. The presence of progeny was scored at 7 days after pairing. Canton-S flies were used in crosses to make $fru^-/+$ control males.

[a] Based on data from this article (Figure 2, Table 2).

[b] While P3 transcripts are detected by RT-PCR in fru^{w27}, these likely supply little or no *fru* P3 function (see text).

additional male-sterile mutations in the vicinity of *fru* on these chromosomes.

Male-male and male-female courtship performed by *fru* mutant males: To gain more insight into *fru*'s male-specific function, we examined various components of male courtship behavior in a number of heteroallelic *fru* mutant combinations. One measure of overall courtship levels is given by the courtship index (CI), which indicates the amount of time a male fly spends courting (see MATERIALS AND METHODS). The wing extension index (WEI) provides a second measure of the courtship (see MATERIALS AND METHODS). Wild-type males court females vigorously, but court males at very low levels, as is seen from the CIs when $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{w24}$, $In(3R)fru^{w27}$, or $Df(3R)fru^{4\text{-}40}$ are heterozygous with a wild-type chromosome (Table 5A). During courtship wild-type males perform courtship song for roughly one-half of the interval spent courting (WEI; Table 5A).

Previous analyses (RYNER *et al.* 1996; GOODWIN *et al.* 2000) had led us to suggest that it was the products of the P1 *fru* transcripts that carried out *fru*'s role in male courtship. To assess the effects on courtship of just-impaired P1 function we examined the effects of $T(3;Y)$-fru^{w9}, $T(3;het)fru^{w12}$, $Df(3R)fru^{sat15}$, and $In(3R)fru^{w27}$ when heterozygous with $Df(3R)Cha^{M5}$ (Table 5B). We also examined the effects of the absence of both P1- and P2-encoded functions on male courtship behavior in $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $In(3R)fru^{w27}$, $Df(3R)fru^{sat15}$, and *Df(3R)P14* when heterozygous with $Df(3R)fru^{4\text{-}40}$ (Table 5C). In all of these genotypes male-female and male-male courtship measured by either the CI or the WEI is almost completely abolished [unlike what is seen in less severe *fru* genotypes (see below)]. Taken together these results indicate that in the absence of *fru*'s P1-encoded functions all aspects of male-male and male-female courtship are abolished.

We also examined male courtship behavior in flies in which the new *fru* alleles were heterozygous with one of the hypomorphic alleles, fru^1, fru^2, fru^3, or fru^4 (Table 5D). The new alleles over either fru^3 or fru^4 were nearly as severe in their effects as the above genotypes in which

TABLE 5

Courtship behavior of *fru* mutant males

Genotypes	CI (m → m)	WEI (m → m)	CI (m → f)	WEI (m → f)	ChI
			A. fru^+ control genotypes		
fru^+/fru^{w9}	7 ± 2 (10)	0 ± 0	85 ± 6 (10)	49 ± 5	0 ± 0 (7)
fru^+/fru^{w12}	1 ± 1 (5)	0 ± 0	85 ± 9 (5)	54 ± 7	0 ± 0 (5)
fru^+/fru^{w24}	6 ± 3 (5)	1 ± 1	84 ± 4 (5)	58 ± 4	0 ± 0 (5)
fru^+/fru^{w27}	1 ± 1 (5)	0 ± 0	94 ± 2 (5)	66 ± 5	0 ± 0 (5)
$fru^+/fru^{4\text{-}40}$	6 ± 2 (5)	0 ± 0	73 ± 13 (6)	37 ± 8	0 ± 0 (5)
			B. *fru* genotypes with P2, P3, and P4 functions		
fru^{w9}/Cha^{M5}	9 ± 4 (13)	0 ± 0	13 ± 7 (15)	6 ± 3	19 ± 5[a] (7)
fru^{w12}/Cha^{M5}	2 ± 0 (15)[b]	0 ± 0[b]	0 ± 0 (10)[b]	0 ± 0[b]	5 ± 3[a] (7)
fru^{w27}/Cha^{M5}	2 ± 2 (15)[b]	0 ± 0[a]	0 ± 0 (10)[b]	0 ± 0[b]	36 ± 10[a] (8)
fru^{sat15}/Cha^{M5}	0 ± 0 (17)	0 ± 0	0 ± 0 (16)	0 ± 0	36 ± 5 (12)
			C. *fru* genotypes with just P3[h] and P4 functions		
$fru^{w9}/fru^{4\text{-}40}$	12 ± 6 (11)	2 ± 2	16 ± 6 (10)	6 ± 3	12 ± 6 (10)
$fru^{w12}/fru^{4\text{-}40}$	8 ± 4 (11)	1 ± 1	3 ± 3 (7)	0 ± 0	1 ± 0 (3)
w, fru^{w12}/fru^{w27}	1 ± 1 (2)	0 ± 0	2 ± 2 (4)	0 ± 0	0 (1)[f]
$w+$, fru^{w12}/fru^{w27}	1 ± 1 (7)	0 ± 0	0 ± 0 (6)	0 ± 0	0 ± 0 (3)[f]
$fru^{w27}/fru^{4\text{-}40}$	10 ± 5 (14)	1 ± 0	4 ± 3 (10)	0 ± 0	21 ± 13 (6)
$fru^{4\text{-}40}/fru^{sat15}$	6 ± 2 (25)	0 ± 0	0 ± 0 (19)	0 ± 0	12 ± 4 (9)
$fru^{4\text{-}40}/P14$	10 ± 5 (15)	0 ± 0	4 ± 3 (19)	0 ± 0	3 ± 2 (7)
			D. *fru* genotypes with hypomorphic *fru* alleles		
fru^{w9}/fru^{1}	46 ± 7 (11)	26 ± 6	68 ± 4 (10)	41 ± 6	54 ± 6 (6)
fru^{w9}/fru^{2}	5 ± 2 (9)	1 ± 0	15 ± 6 (10)	9 ± 4	25 ± 8[g] (11)
fru^{w9}/fru^{3}	1 ± 0 (13)	0 ± 0	1 ± 1 (14)	0 ± 0	31 ± 8 (7)
fru^{w9}/fru^{4}	9 ± 5 (13)	1 ± 1	0 ± 0 (11)	0 ± 0	32 ± 7 (15)
fru^{w12}/fru^{1}	47 ± 8 (12)	21 ± 4	36 ± 9 (11)	17 ± 5	41 ± 6 (7)
fru^{w12}/fru^{2}	3 ± 1 (12)	0 ± 0	5 ± 2 (13)	2 ± 2	15 ± 4 (10)
fru^{w12}/fru^{3}	0 ± 0 (14)[b]	0 ± 0[b]	1 ± 1 (11)[b]	0 ± 0[b]	11 ± 4[c] (10)
fru^{w12}/fru^{4}	8 ± 5 (9)[b]	1 ± 1[b]	7 ± 5 (12)[b]	0 ± 0[b]	19 ± 4 (4)
fru^{w24}/fru^{1}	46 ± 6 (10)	12 ± 3	42 ± 9 (12)	15 ± 4	58 ± 8 (10)
fru^{w24}/fru^{2}	4 ± 2 (12)	0 ± 0	11 ± 3 (13)	2 ± 2	7 ± 2[d] (8)
fru^{w24}/fru^{3}	13 ± 6 (8)	1 ± 0	0 ± 0 (10)	0 ± 0	16 ± 7[d] (8)
fru^{w24}/fru^{4}	15 ± 7 (8)	1 ± 1	16 ± 8 (8)	1 ± 1	25 ± 5[d] (12)
fru^{w27}/fru^{1}	30 ± 10 (11)	16 ± 6	40 ± 11 (10)	24 ± 7	47 ± 8 (11)
fru^{w27}/fru^{2}	3 ± 3 (10)	0 ± 0	17 ± 8 (13)	2 ± 1	7 ± 4[e] (12)
fru^{w27}/fru^{3}	8 ± 6 (11)	1 ± 0	0 ± 0 (13)	0 ± 0	13 ± 7[e] (7)
fru^{w27}/fru^{4}	11 ± 4 (14)	1 ± 1	0 ± 0 (12)	0 ± 0	36 ± 5 (13)
$fru^{4\text{-}40}/fru^{1}$	44 ± 5 (10)	18 ± 5	33 ± 7 (10)	13 ± 4	46 ± 8 (10)
$fru^{4\text{-}40}/fru^{2}$	30 ± 9 (10)	15 ± 6	26 ± 10 (10)	15 ± 7	27 ± 6 (9)
$fru^{4\text{-}40}/fru^{3}$	26 ± 8 (10)	0 ± 0	1 ± 1 (11)	0 ± 0	31 ± 7 (8)
$fru^{4\text{-}40}/fru^{4}$	22 ± 9 (10)	3 ± 2	6 ± 4 (11)	0 ± 0	29 ± 7 (7)

Males to be tested for their courtship behavior were collected just after eclosion and aged individually for 6–10 days. The test male was placed in a small observation chamber with either another male of the same genotype or a virgin Canton-S female. The courtship index (CI ± SEM) is the percentage of time that a male spends courting another fly, male (CI m → m) or female (CI m → f), during the observation period. When the male is paired with another male, the CI is calculated only for the first male to initiate courtship toward the other male. When the male is paired with a female, the CI represents the time that the male courts the female. The wing extension index (WEI ± SEM, Villella *et al.* 1997) is the amount of time that the male extended his wing during the observation period. The number of males tested for CI and the WEI is given in parentheses in the CI column. A one-way ANOVA with group as the main effect revealed significant differences among groups [$F_{(65, 591)} = 16.78$, $P < 0.0001$]. Each group is represented by the CI value given in each cell of Table 5. Subsequent planned comparisons (P deemed significant if $<\alpha = 0.004$) to determine if heterozygous controls were different in levels of courtship toward males *vs.* females revealed that all the heterozygous controls were the same. Hence, these four groups (fru^+/fru^{w9}, fru^+/fru^{w12}, fru^+/fru^{w24}, and fru^+/fru^{w27}) were combined into one control group for males courting males and a second control group for males courting females. A second one-way ANOVA with group as the main effect revealed significant differences among groups [$F_{(59,597)} = 18.43$, $P < 0.0001$]. Subsequent planned comparisons (P significant if $<\alpha = 0.002$) between control males (consisting of all heterozygous types mentioned above) and mutant males courting other males showed that

fru's P1 function was absent: Male-female courtship as measured by the CI or WEI was abolished as was male-male courtship as measured by WEI. However, in some genotypes, most notably $Df(3R)fru^{4\text{-}40}/fru^{3}$ and $Df(3R)fru^{4\text{-}40}/fru^{4}$, there was significant male-male courtship as measured by the CI. The effects of the new alleles over fru^{2} were also severe: male-male courtship was essentially abolished as measured by either the CI or WEI, as was male-female courtship as measured by the WEI. In most genotypes involving fru^{2}, measurable male-female courtship was produced. Courtship behavior of males carrying one of the new *fru* alleles over fru^{1} with either a male or female was substantial as measured by both CI and WEI. These males had CIs similar to those of fru^{1} homozygote males (Villella *et al.* 1997). Interestingly, these fru^{1} heterozygous combinations are almost all sterile (Table 4B). In all fru^{1} heterozygous combinations male-female courtship was less frequent than that seen with wild-type males (Table 5D *vs.* 5A); this may be partially due to these mutant individuals being in general less active than wild type (see below).

In summary, heteroallelic combinations of all of the new *fru* mutant alleles with either fru^{2}, fru^{3}, fru^{4}, $Df(3R)fru^{4\text{-}40}$, or $Df(3R)Cha^{M5}$ were not significantly different from wild-type males in their courtship of other males, showing that they no longer had the strong male-male courtship behavior associated with fru^{1} or homozygosity for the *P*-element *fru* alleles (Table 5; Villella *et al.* 1997). Thus these *fru* males are significantly different from wild-type males in that they do not court females and different from fru^{1}, fru^{3}, and fru^{4} homozygotes since they do not court males and females. These findings also show that the reduction in wing extension first observed in fru^{3} and fru^{4} homozygotes and *trans*-heterozygotes (Villella *et al.* 1997) is common to other severely affected *fru* genotypes as well.

Although most *fru* males that court respond to males and females roughly equally, several previously studied *fru* mutant genotypes showed a courtship bias toward either males or females. For example, fru^{3} males courted males more vigorously than females while fru^{2} males courted females more avidly than males (Gailey and Hall 1989; Villella *et al.* 1997). We therefore asked whether these new *fru* mutants showed any consistent bias in the courtship of males *vs.* females. Males from twenty-four *fru* mutant genotypes courted males and females approximately equally (Table 5). Overall, the CIs of males of a particular *fru* genotype to male or female targets are highly correlated [$CI_{m\text{-}m}$ compared to $CI_{m\text{-}f}$: $F_{(29,\ 4586)} = 64.9$, correlation coefficient = 0.83, $R^2 = 69.1\%$; linear regression analysis, Statgraphics 5.0], suggesting that *fru* males are not discriminating between

TABLE 5

(Continued)

only fru^{1} mutants heterozygous with fru^{w9}, fru^{w12}, fru^{w24}, fru^{w27}, and $fru^{4\text{-}40}$, as well as fru^{3}/fru^{w24}, courted males significantly more than controls (all P's $<\alpha = 0.002$). All other combinations were not different from controls in CI levels for males courting males. Planned comparisons between control males and mutant males courting females showed that all *fru* mutant males, except for fru^{1}/fru^{w9}, courted females significantly less than controls (all P's < 0.002). To determine the degree of male-male courtship chain formation, males were aged individually for 5–6 days, and then eight males were grouped together in a food vial (day 1). The number of replicates is given in parentheses. Observations were made at day 3 or 4 after grouping. A chaining index (ChI) was calculated as the percentage of time three or more males were in a chain formation for a given 10-min observation period (±SEM). A one-way ANOVA with genotype as the main effect revealed significant differences among genotypes [$F_{32,230} = 8.24$, $P < 0.0001$]. Subsequent planned pairwise comparisons (P significant if $<\alpha = 0.009$) to determine whether heterozygous controls were different in levels of chaining revealed that all heterozygous combinations were the same in ChIs (all P's ≥ 0.009). Hence, these heterozygous combinations were all grouped into one control group. A second one-way ANOVA with genotype (including new control group) as the main effect revealed significant differences among genotypes [$F_{(29,233)} = 9.21$, $P < 0.0001$]. Subsequent comparisons between ChIs of mutant males compared to controls were performed using Dunnett's test and were deemed significant at 5%. The following genotypes had chaining levels that were not significantly different (all P's ≥ 0.05) from controls: fru^{w12}/Cha^{M5}, $fru^{w12}/fru^{4\text{-}40}$, w^{+}; fru^{w12}/fru^{w27}, fru^{w24}/fru^{2}, fru^{w27}/fru^{2}, fru^{w27}/fru^{3}, and $fru^{4\text{-}40}/P14$. Further planned comparisons among genotypes (P significant if $<\alpha = 0.003$) are summarized in footnotes a–e.

[a] fru^{w27}/Cha^{M5} males showed significantly higher chain formation than fru^{w12}/Cha^{M5} ($P < \alpha = 0.003$) but were not different from fru^{w9}/Cha^{M5} ($P > \alpha = 0.003$).

[b] Data from Ryner *et al.* (1996).

[c] fru^{3}/fru^{w12} males had ChIs that were lower than those of fru^{1}/fru^{w12} ($P < \alpha = 0.003$).

[d] Heterozygous fru^{2}/fru^{w24}, fru^{3}/fru^{w24}, and fru^{4}/fru^{w24} males showed significantly less chaining than fru^{1}/fru^{w24} males (all P's $< \alpha = 0.003$).

[e] fru^{2}/fru^{w27} and fru^{3}/fru^{w27} showed chaining levels that were different from those of fru^{1} over the same breakpoint (all P's $< \alpha = 0.003$).

[f] fru^{w12}/fru^{w27} ChIs were calculated with only four to five males per group since these males are few in numbers.

[g] fru^{2}/fru^{w9} showed ChIs that were significantly lower than those of fru^{1}/fru^{w9} ($P < \alpha = 0.003$).

[h] While P3 transcripts are detected by RT-PCR in fru^{w27}, these likely supply little or no *fru* P3 function (see text).

TABLE 6

Short-term activity of *fru* males

Genotype	Activity ± SEM	Genotype	Activity ± SEM
	A. fru^+ control genotypes		
fru^+/fru^+	77 ± 8 (15)[a]	fru^+/fru^{w24}	78 ± 11 (10)
fru^+/fru^{w9}	42 ± 3 (25)	fru^+/fru^{w27}	78 ± 5 (17)
fru^+/fru^{w12}	63 ± 7 (10)	$fru^+/fru^{4\text{-}40}$	79 ± 8 (10)
fru^+/Cha^{M5}	65 ± 4 (20)	$fru^+/P14$	49 ± 4 (14)
fru^+fru^{sat15}	74 ± 4 (18)		
	B. *fru* genotypes with little or no P1, or P1 and P2 function		
Cha^{M5}/fru^{w9}	46 ± 8 (17)	Cha^{M5}/fru^{w27}	54 ± 8 (8)
Cha^{M5}/fru^{w12}	28 ± 5 (22)	Cha^{M5}/fru^{sat15}	43 ± 6 (14)
$fru^{4\text{-}40}/fru^{w9}$	38 ± 7 (18)	$fru^{4\text{-}40}/P14$	51 ± 7 (14)
$fru^{4\text{-}40}/fru^{w12}$	13 ± 5 (13)	$w; fru^{w12}/fru^{w27}$	7 ± 4 (11)
$fru^{4\text{-}40}/fru^{w27}$	18 ± 4 (12)	$w^+; fru^{w12}/fru^{w27}$	4 ± 1 (17)
$fru^{4\text{-}40}/fru^{sat15}$	36 ± 5 (20)		
	C. *fru* genotypes involving hypomorphic alleles		
fru^1/fru^{w9}	36 ± 7 (10)	fru^3/fru^{w9}	51 ± 8 (10)
fru^1fru^{w12}	41 ± 8 (11)	fru^3/fru^{w12}	62 ± 7 (20)
fru^1/fru^{24}	31 ± 7 (11)	fru^3/fru^{w24}	44 ± 5 (14)
fru^1/fru^{w27}	73 ± 11 (11)	fru^3/fru^{w27}	71 ± 16 (10)
$fru^1/fru^{4\text{-}40}$	48 ± 7 (11)	$fru^3/fru^{4\text{-}40}$	80 ± 5 (10)
fru^2/fru^{w9}	13 ± 3 (15)	fru^4/fru^{w9}	55 ± 4 (13)
fru^2/fru^{w12}	8 ± 5 (10)	fru^4/fru^{w12}	64 ± 9 (11)
fru^2/fru^{w24}	19 ± 5 (10)	fru^4/fru^{w24}	58 ± 5 (14)
fru^2/fru^{w27}	24 ± 5 (13)	fru^4/fru^{w27}	95 ± 12 (13)
$fru^2/fru^{4\text{-}40}$	53 ± 3 (10)	$fru^4/fru^{4\text{-}40}$	81 ± 6 (8)

Males were placed in a chamber that is divided by a line in the middle. The male was allowed to rest for 2–3 min, then the number of times the male crossed the line during a 3-min observation period was counted using a hand-held timer. The numbers above are the average number of crossings/3-min observation period ± SEM. The numbers in parentheses are the total number of observations. For the statistical analysis of short-term activity, a one-way ANOVA with genotype as the main effect revealed significant differences among genotypes [$F_{(35,406)} = 12.14$, $P < 0.0001$]. Subsequent comparions (Dunnett's test) between the above *fru* variants and the wild-type control (Canton-S) revealed that the following genotypes (identified by underlining) were significantly different (all P's < 0.05) in short-term activity compared with wild type. To explore whether there was any relationship between the courtship and activity phenotypes of these different genotypes, the correlation between the CI for male-male and male-female courtship *vs.* the mean short-term activitiy was calculated (short-term activity compared to $CI_{m\text{-}m}$; $F_{(29,353)} = 1.63$, correlation coefficient = 0.23; $R^2 = 5.3\%$. Short-term activity compared to $CI_{m\text{-}f}$: $F_{(29,8.35)} = 0.03$, correlation coefficient = 0.03; $R^2 = 0.10\%$, linear regression analysis, SAS).

[a] Data from Villella *et al.* (1997).

male and female partners independent of whatever level of courtship they generate.

Short-term activity of *fru* mutant males: One explanation for the failure of *fru* males to court is that these males might be generally inactive or sluggish (for review, see Hall 1994). To evaluate the general behavioral robustness of the new *fru* genotypes, a short-term activity assay was used to measure the voluntary activity a single male performs in the same chamber used for the courtship assays (Villella *et al.* 1997). Tests of five genotypes that do not express P1 or P2 *fru* transcripts [$Df(3R)fru^{4\text{-}40}$ heterozygous with either $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{sat15}$, $In(3R)fru^{w27}$, or $Df(3R)P14$] and four genotypes that have little or no functional P1 products [$Df(3R)Cha^{M5}$ heterozygous with either $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{w27}$, or $Df(3R)fru^{sat15}$] showed that nearly all of these genotypes had reduced short-term activity compared to fru^+ control males (Table 6, B *vs.* A). However, two of these genotypes [$Df(3R)fru^{4\text{-}40}/Df(3R)P14$ and $Df(3R)Cha^{M5}/In(3R)fru^{w27}$] had wild-type activity levels, suggesting that the reduced activity seen in the other genotypes may be due to background effects rather than the result of reduced *fru* expression.

We also examined short-term activity in males heteroallelic for one of the new *fru* alleles and either fru^1, fru^2, fru^3, or fru^4. Heteroallelic combinations with fru^3 or fru^4 had, with one exception, wild-type levels of activity (Table 6C). However, all heteroallelic combinations of one of the new alleles with fru^2 showed greatly reduced short-term activity (Table 6C). Since all of the fru^{w-} alleles tested were induced on a fru^2 parental chromosome, the reduced activity seen in these genotypes likely represents the effects of this common genetic background. In this regard it is worth noting that $In(3R)fru^{w27}/(T(3;het)$

fru^{w12} escapers (this is an essentially lethal genotype; Table 3) also show low levels of activity.

Overall, almost one-half of the *fru* heteroallelic combinations tested were significantly less active than wild-type males (Table 6, B and C). However, there is only a weak correlation between the level of short-term activity for these *fru* mutant males and the mean CIs they produce for either male-male or male-female courtship (Table 6 legend). These findings suggest that the failure of *fru* mutant males of these genotypes to court prospective mates in pairwise tests is predominantly a specific courtship defect rather than simply the result of these males being less active.

Male-male group courtship (chaining) by *fru* mutant males: *fru* mutant males exhibit a dramatic behavioral phenotype, the formation of male-male courtship chains (Hall 1978; Gailey and Hall 1989; Gailey *et al.* 1991; Villella *et al.* 1997). Males homozygous for fru^{1} court each other in groups, from a young-adult age onward (Villella *et al.* 1997). For other genotypes, such as fru^{3} and fru^{4}, significant chaining is seen only after the males have been grouped together for a few days (Villella *et al.* 1997). A ChI, measuring the time that three or more males were courting during the observation period, was calculated to quantify the amount of intermale courtship produced by the different genotypes we examined (Table 5).

Males carrying one of the new *fru* alleles [$T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{w24}$, $In(3R)fru^{w27}$, and $Df(3R)fru^{4\text{-}40}$] over one of the hypomorphic *fru* alleles (fru^{1}, fru^{2}, fru^{3}, and fru^{4}) exhibited substantial chaining (Table 5D). Chaining was highest in fru^{1} heteroallelic combinations, somewhat less in fru^{3} and fru^{4} heteroallelic combinations, and lower still in fru^{2} heteroallelic combinations. This parallels the levels of chaining seen in homozygotes for these four hypomorphic alleles (Villella *et al.* 1997). While the levels of chaining in fru^{1} heteroallelic combinations are lower than that seen in fru^{1} homozygotes [41–58% (Table 5D) *vs.* ~70% (Villella *et al.* 1997)], in the cases of the fru^{2}, fru^{3}, and fru^{4} heteroallelic combinations the levels of chaining are, in general, comparable to those previously reported for the respective homozygous mutants (Villella *et al.* 1997).

Chaining was also examined in four heteroallelic *fru* combinations that have little or no functional P1 *fru* promoter-derived products (Table 5B) and six heteroallelic combinations that have no functional P1 or P2 *fru* promoter-derived products (Table 5C). The results were quite variable with four genotypes [$T(3;het)fru^{w12}/Df(3R)Cha^{M5}$, $T(3;het)fru^{w12}/Df(3R)fru^{4\text{-}40}$, $T(3;het)fru^{w12}/In(3R)fru^{w27}$, and $Df(3R)fru^{4\text{-}40}/Df(3R)P14$], showing no chaining, while the remaining five genotypes [$T(3;Y)fru^{w9}/Df(3R)Cha^{M5}$, $In(3R)fru^{w27}/Df(3R)Cha^{M5}$, $T(3;Y)fru^{w9}/Df(3R)fru^{4\text{-}40}$, $In(3R)fru^{w27}/Df(3R)fru^{4\text{-}40}$, and $Df(3R)fru^{4\text{-}40}/Df(3R)fru^{sat15}$] showed significant levels of chaining. We do not understand the bases for these differences between ostensibly equivalent *fru* genotypes. Three of the four genotypes that did not chain are less active than wild type (Table 6), but one of the *fru* mutants that did not chain, $Df(3R)fru^{4\text{-}40}/Df(3R)P14$, had high levels of short-term activity. On the other hand, some mutant genotypes with low mean short-term activity [$In(3R)fru^{w27}/Df(3R)fru^{4\text{-}40}$ and $T(3;Y)fru^{w9}/fru^{2}$] still performed moderate levels of chaining (Tables 5 and 6).

To determine whether there was a general relationship between the level of short-term activity and chaining behavior by *fru* mutant males, the correlation between the mean ChI *vs.* the mean short-term activity measurement was calculated for all genotypes (correlation coefficient = 0.41, R^2 = 17.2%; linear regression analysis, Statgraphics 5.0). It is apparent from the regression analysis that there is a correlation between the level of short-term activity of flies and their ChI. Nevertheless, these findings do suggest that a part of the reduction in chaining levels represents the loss of a *fru*-dependent function and that the reduction in ChI is not solely due to the reduced activity of the participating male flies.

In the analysis of the courtship behavior of these *fru* mutant males, we noted that males of several *fru* genotypes that had essentially no courtship to either males or females in pairwise tests still showed male-male chaining behavior at moderate to high levels (Table 5). To better analyze the relationship between the different measures of courtship activity, a linear regression analysis was carried out. It shows that there is a strong positive correlation between the mean $CI_{m\text{-}m}$ and the ChI [$F_{(29,\ 3590)}$ = 34.18, correlation coefficient = 0.74; R^2 = 54.1%; linear regression analysis, SAS). Seven *fru* mutant genotypes [*e.g.*, $Df(3R)fru^{4\text{-}40}/Df(3R)P14$, $T(3;het)fru^{w12}/Df(3R)fru^{4\text{-}40}$, $T(3;het)fru^{w12}/Df(3R)Cha^{M5}$], which showed no chaining behavior, also did not court males or females, suggesting that the loss of the chaining behavior occurred separately and subsequently to the loss of all of the individual courtship behaviors as measured in courtship tests and that the absence of chaining is an indication of a more severe loss of *fru* function.

Expression of the courtship song by *fru* mutant males: Wild-type males produce a dual component song consisting of a hum or sine song and a pulse song composed of a train of pulses, which is amenable to detailed quantitative analysis (Wheeler *et al.* 1988, 1989; Bernstein *et al.* 1992). Control $fru^{w-}/+$ males generated normal courtship song except that the width of the fast-Fourier transform (FFT) was smaller in the control males *vs.* wild type (Table 7A). $fru^{1\text{-}4}/+$ controls also showed a similar change in the width of the FFT compared to wild-type males (Villella *et al.* 1997).

Males carrying one of the new *fru* alleles over fru^{1} produced a courtship song that had longer interpulse intervals (IPIs), but essentially normal intrapulse frequencies, cycles per pulse, and width of the FFT when compared to the control males (Table 7B).

Previous experiments had shown that males mutant for some *fru* alleles, such as fru^{3}/fru^{1}, produced a court-

TABLE 7

Courtship song summary for *fru* mutants

Genotype	*N* wing extension	*N*	IPI	CPP	Frequency	Width
			A. fru^{+} control genotypes			
+/+[a]	5	5	0.037 ± 0.001	2.66 ± 0.19	258 ± 9	180 ± 10
fru^{w9}/+	5/10	4	0.037 ± 0.001	2.80 ± 0.08	232 ± 10	168 ± 19
fru^{w12}/+	3	3	0.034 ± 0.001	2.52 ± 0.17	250 ± 8	149 ± 4
fru^{w24}/+	3	3	0.034 ± 0.001	2.73 ± 0.12	240 ± 5	142 ± 7
fru^{w27}/+	3	3	0.036 ± 0.001	2.94 ± 0.28	226 ± 3	126 ± 7
			B. *fru* genotypes involving hypomorphic alleles			
fru^{1}/fru^{w9}	20/21	5	0.043 ± 0.001	3.68 ± 0.14	245 ± 7	160 ± 10
fru^{1}/fru^{w12}	20/24	5	0.043 ± 0.003	4.08 ± 0.29	240 ± 5	134 ± 10
fru^{1}/fru^{w24}	19/21	5	0.048 ± 0.004	3.25 ± 0.45	233 ± 3	117 ± 8
fru^{1}/fru^{w27}	15/21	5	0.046 ± 0.002	3.56 ± 0.23	244 ± 11	130 ± 12
$fru^{1}/fru^{4\text{-}40}$	4/4	4	0.049 ± 0.005	4.68 ± 0.34	272 ± 21	119 ± 11
fru^{3}/fru^{w9}	0 (27)	0	NA	NA	NA	NA
fru^{3}/fru^{w12}	0 (25)	0	NA	NA	NA	NA
fru^{3}/fru^{w24}	4 (15)	0 (4)	—	—	—	—
fru^{3}/fru^{w27}	2/20	0 (2)	—	—	—	—
$fru^{3}/fru^{4\text{-}40}$	1/3	0 (1)	—	—	—	—
fru^{4}/fru^{w9}	2/14	1 (2)[b]				
fru^{4}/fru^{w12}[b]	4/21	1 (4)[b,c]				
fru^{4}/fru^{w24}	7/16	0 (7)	—	—	—	—
$fru^{4}fru^{w27}$	2/24	1 (2)[d]				
$fru^{4}/fru^{4\text{-}40}$	3/18	0 (3)	—	—	—	—
			C. *fru* genotypes with little or no P1, or P1 and P2 function			
$fru^{4\text{-}40}/fru^{w9}$[b]	10/21	1 (10)[b,e]	0.056	3.96	274	119
$fru^{4\text{-}40}/fru^{w12}$	4/18	0 (4)	—	—	—	—
$fru^{4\text{-}40}/fru^{w27}$	5/11	0 (5)	—	—	—	—
Cha^{M5}/fru^{w9}	4/29	4	0.036 ± 0.004	3.80 ± 0.10	305 ± 22	227 ± 22
Cha^{M5}/fru^{w12}	4/20	0 (4)	—	—	—	—

Quantitative analysis of the pulse component of the courtship song for *fru* mutant and control males. The column head "*N* wing extension" shows the number of males who produced wing extension of those tested. Those genotypes, which did not show any courtship and therefore produced no song, are denoted by NA. The next column, *N*, shows the number of males who extended their wing and produced courtship song. The *fru* mutant genotypes where males extended their wing but did not produce any courtship song have a zero in the *N* column and the absence of song data is shown as dashes in the following columns. In some genotypes, males produced only sine song; therefore, no pulse trains could be analyzed (as indicated by dashes, see details below). In those genotypes where males produced pulse trains, only those trains with >3 pulses/train and the interval between pulses was <100 ms were quantitatively analyzed. At least 15–20 trains were analyzed for most genotypes; fewer trains were used for those genotypes that vibrate their wings more weakly (see below). The song parameters that were analyzed are as follows: CPP, the number of cycles per pulse given as mean ± SEM; IPI, the interpulse interval, which is represented by the mean; and, width, the width of the fast Fournier transform of the song in hertz. The SEMs for the IPI values are not shown but all were within 0.001–0.005. In several genotypes, only a fraction of the males produced a pulse song that could then be analyzed.

[a] From Villella *et al.* (1997).

[b] In these mutants, fewer than six trains were analyzed.

[c] Although four fru^{4}/fru^{w12} males showed wing extension, only two produced sound. One male generated only sine song and the other one produced pulse song while extending both wings. The other two did not produce any sound.

[d] One of the fru^{4}/fru^{w27} males that produced wing extension only showed two brief bouts of sine song and the other one produced pulse song extending both wings.

[e] Out of the 10 $fru^{4\text{-}40}/fru^{w9}$ flies that extended their wing, four produced sound; three of these males only produced sine song and one male produced pulse song that could be analyzed. The remaining six males did not sing at all.

TABLE 8

***fru* mutations disrupt the muscle of Lawrence development**

First chromosome *3*, *fru* genotype	Second chromosome *3*, *fru* genotype				(First chromosome *3*, homozygous)
	fru^{w9}	fru^{w12}	fru^{w24}	fru^{w27}	
fru^{1}	11, 2, 2, 1, 4	7, 6, 4, 2, 1	9, 3, 6, 2, 0	5, 4, 8, 3, 0	(6, 1, 11, 2, 0)[a]
fru^{2}	2, 3, 3, 6, 6	1, 5, 6, 5, 3	4, 5, 9, 1, 1	5, 7, 2, 4, 2	(0, 0, 0, 10, 10)[a]
fru^{3}	20, 0, 0, 0, 0	20, 0, 0, 0, 0	19, 1, 0, 0, 0	18, 2, 0, 0, 0	(19, 1, 0, 0, 0)[b]
fru^{4}	19, 1, 0, 0, 0	18, 1, 1, 0, 0	19, 1, 0, 0, 0	18, 2, 0, 0, 0	(20, 0, 0, 0, 0)[b]
$Df(3R)Cha^{M5}$	16, 4, 0, 0, 0	18, 2, 0, 0, 0	Lethal	18, 2, 0, 0, 0	(Lethal)
$fru^{4\text{-}40}$	20, 0, 0, 0, 0	19, 1, 0, 0, 0	Lethal	19, 1, 0, 0, 0	(Lethal)
fru^{+}	0, 0, 0, 0, 8[c]	0, 0, 0, 0, 8[c]	0, 0, 0, 0, 8[c]	0, 0, 0, 0, 8[c]	(0, 0, 0, 0, 20)[a]

The new *fru* mutants fail to complement previously reported *fru* alleles and aberrations for MOL development. Since the MOL is a bilaterally symmetrical structure, for a given fly, each "half" of the MOL was given a separate phenotypic score; thus each fly generated two data points. Phenotypic scores for a given genotype were then summed, $n = 10$ males per genotype, yielding a total of 20 data points per genotype block (for combinations with fru^{+}, $n = 4$ males dissected). The phenotypic classes are as reported in Villella *et al.* (1997) and range, reading from left to right within a given block, from "Abs" (no MOL development on a given side—the "MOL absent" class of Villella *et al.* (1997)—through intermediate phenotypes "a-1", "a-2", "b", to "+", or normal MOL development on a given side. The genotype fru^{1}/fru^{w9} is explained as example: In total, 11 "halves" showed no MOL-like development, 2 were "a-1", 2 were "a-2", 1 was "b", and 4 were "wild type" in appearance. Thus the larger the numbers on the right of the score, the more normal the MOL expression (note the 0, 0, 0, 0, 20 score for fru^{+}/fru^{+}, indicating all MOLs normal). Conversely, the larger the numbers on the left side of the score the more mutant the MOL expression (note the 20, 0, 0, 0, 0 score for fru^{3}/fru^{w9}, indicating all MOLs absent). Two females were dissected for all adult-viable genotypes and no MOL-like development was observed (crosses with fru^{w9} yield no females; see MATERIALS AND METHODS). MOL scores for homozygous-viable *fru* alleles are provided in the rightmost column.

[a] New data set, this article. See discussions in Villella *et al.* (1997) regarding viable MOL expression associated with the fru^{1} and fru^{2} alleles.

[b] Same dissections as reported in Villella *et al.* (1997).

[c] $n = 4$ flies dissected for these control genotypes.

ship song, but homozygous fru^{3} males or $fru^{3}/P14$ males did not (Ryner *et al.* 1996; Villella *et al.* 1997). We examined the song production of males carrying heteroallelic combinations of the new *fru* alleles with either fru^{3} or fru^{4} as a further test for the severity of the new *fru* alleles. These males did not produce a courtship song even though they did produce a small amount of wing extension (Tables 5D and 7B). Other heteroallelic combinations with either $Df(3R)Cha^{M5}$ or $Df(3R)fru^{4\text{-}40}$, which had very reduced wing extension, also failed to produce courtship song during the times that they did extend their wing (Table 7C). These findings indicate that the new mutants are stronger alleles of *fru* and that courtship song is dependent on P1 function.

MOL of *fru* mutant males: *fru* was previously shown to control the male-specific differentiation of the MOL (Gailey *et al.* 1991). Five of the new *fru* mutations were tested in combination with previously reported *fru* mutations or deletions for their effects on MOL formation (Table 8). All viable combinations of $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $Df(3R)fru^{w24}$, and $In(3R)fru^{w27}$ with fru^{3}, fru^{4}, $Df(3R)Cha^{M5}$, and $Df(3R)fru^{4\text{-}40}$ failed to make a normal-sized MOL; all of the muscle fibers were small and the same size as nearby longitudinal muscles. This represents one of the strongest *fru*-associated MOL phenotypes (Gailey *et al.* 1991; Taylor and Knittel 1995; Villella *et al.* 1997; B. J. Taylor and L. Knittel, unpublished results).

Trans-heterozygotes between $Df(3R)Cha^{M5}$ and $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, or $In(3R)fru^{w27}$ showed almost no MOL development (Table 8). The slight MOL development noted in these genotypes likely stems from the *ad hoc* distinction of an occasional MOL showing some coalescence of the small longitudinal fibers at the expected fifth-abdominal-tergite site of MOL organization, the "a-1" category of Villella *et al.* (1997). It is not clear that these infrequent observations of marginal fiber clustering correlate with *fru* gene expression. Thus these results indicate that P1-derived functions are also essential for MOL development.

$T(3;Y)fru^{9}$, $T(3;het)fru^{12}$, or $In(3R)fru^{w27}$ in combination with the hypomorphic alleles fru^{1}, fru^{2}, fru^{3}, or fru^{4} had varying effects on MOL development. The fru^{3} and fru^{4} combinations showed the most severe disruption and were indistinguishable from the $Df(3R)Cha^{M5}$ combinations (MOLs missing and sterile; Table 8 and Table 4B). However, the fru^{1} and fru^{2} combinations showed an interesting difference. Whereas these combinations also led to sterility (except for the marginal case of $fru^{2}/T(3;Y)fru^{w9}$; Table 4E), they resulted in an appreciable

amount of intermediate-to-complete MOL development (Table 8). These results confirm that among all *fru* alleles, the fru^1 and fru^2 mutations have the weakest impact on the MOL, with fru^1 homozygotes showing a variably intermediate phenotype that is not exacerbated when heterozygous with any other *fru* allele, while fru^2 homozygotes develop a nearly wild-type MOL (rows 1 and 2, Table 8; Villella *et al.* 1997).

Characterization of *fru*'s essential functions

Lethal phase: To define the development stages when *fru*'s vital functions are needed and the biological roles of the P3 and P4 transcripts, we examined animals from seven *fru* mutant genotypes chosen to reflect the range of potential lethal phases. Animals of five *fru* genotypes, which lacked P1, P2, and P3 function, such as *T(3;het)*-fru^{w12}/*Df(3R)*fru^{sat15} and *T(3;het)*fru^{w12}/*Df(3R)*fru^{w24}, reached the adult stage but were generally unable to eclose from the pupal case (Tables 9A, 3C, and 3D). On close inspection, both males and females that died within the pupal case had attempted to eclose. These animals were able to successfully emerge as adults if dissected from the pupal case (Table 9A). Such freed animals performed posteclosion behaviors, such as wing and proboscis expansion, but were unable to fully extend their wings, which projected from the body at a 70–90° angle (Figure 3A). Once they emerged, they routinely survived for 7–14 days. By a number of phenotypic criteria, *T(3;het)*-fru^{w12}/*Df* animals were more severely affected than the other genotypes from which survivors were obtained: Fewer *T(3;het)*fru^{w12}/*Df* animals reached the adult stages, and they had more severe anatomical phenotypes (see below) and shorter survival times.

Since there are currently no genotypes that are null for just *fru*, we examined the lethal phases of three genotypes [*Df(3R)*fru^{sat15}/*Df(3R)*fru^{sat15}, *Df(3R)*fru^{sat15}/*Df(3R)P14*, and *Df(3R)*fru^{sat15}/*Df(3R)*fru^{w24}] that are null for *fru* but that also delete a small number of adjacent genes as these genotypes provide an upper limit on the severity and nature of a *fru* null phenotype (Table 9B). These three *fru* genotypes died after forming prepupae (Figure 3D, Table 9B). *Df(3R)*fru^{sat15} is a small deficiency that deletes all *fru* coding sequences as well as all or part of the six genes proximal to *fru*. *Df(3R)*fru^{w24} and *Df(3R)P14* are larger deficiencies that encompass *Df(3R)*fru^{sat15} and so these three genotypes should be equivalent in terms of the genes they delete. Since *Df(3R)*fru^{sat15} homozygotes have a slightly earlier lethal phase than either *Df(3R)*fru^{sat15}/*Df(3R)P14* or *Df(3R)* fru^{sat15}/*Df(3R)*fru^{w24}, this must be due to other lesions on the *Df(3R)*fru^{sat15} chromosome. In the most severely affected cases *fru* prepupae were tanned and frequently formed a posterior bubble but did not have everted anterior spiracles or retracted anterior segments, thus exhibiting some characteristics associated with wandering third instar larvae (Bainbridge and Bownes 1981; Figure 3D; Table 9B). To determine whether these mutants had a defined lethal phase or whether a fraction of the larvae simply died at each molt, we collected 51 first instar *Df(3R)*fru^{sat15}/*Df(3R)*fru^{w24} larvae and monitored their progression through the other larval stages into early pupal development. All animals reached the prepupal stage, suggesting that this pupal lethal phase was specific. The early pupal lethality of these mutants is the most extreme phenotype that is potentially associated with the loss of the *fru* function. Two other genotypes, *Df(3R)*$fru^{4\text{-}40}$/*Df(3R)*fru^{w24} and *Df(3R)*fru^{w24}/*Df(3R) P14*, deleted for larger regions encompassing *fru*, were examined and found to die at the second to third larval molt (data not shown; Table 3). It is likely that the larval lethal phase in these genotypes is due to the absence of other vital gene(s) located in the large regions flanking the *fru* locus uncovered by these deficiencies. Thus, the lethal phase associated with loss of *fru* function is during metamorphosis.

The adult escapers or released animals from these *fru* genotypes all had minor defects in their external appearance (Table 9C). In a high proportion of these mutant escapers, one or more imaginal discs failed to evert and after emergence it was common for one or both wings to not be fully expanded. When the wings were inflated, they were abnormally positioned, being held at a 70–90° angle from the body (Figure 3A; Table 9C). Furthermore, the femur was frequently bent and one or more joints were defective in at least one leg per animal, usually the femur/tibial or the tibial/first tarsal joint in the metathoracic leg (Figure 3B, Table 9C). These defective joints were enlarged and frequently had additional bristles. Based on cuticle preparations made of mutant flies, males and females had duplicated sternopleural and anterior scutellar macrochaetes (Table 9C). These anatomical defects were not found in the fully viable *fru* mutant combinations *Df(3R)*Cha^{M5}/*In(3R)*fru^{w27} (male, $n = 4$; female, $n = 3$) and fru^1/*Df(3R)*fru^{w24} (male, $n = 5$; female, $n = 18$; Figure 3C; data not shown).

Neuronal phenotypes of *fru* mutants: The inability of some of these new *fru* mutants to emerge from the pupal case led us to examine motorneuronal synapses on abdominal muscles to determine whether all muscles were innervated and whether their synaptic morphology was normal. Innervation was assessed by labeling abdominal carcasses with antibodies to the synapse-specific protein, synaptotagmin (Littleton *et al.* 1993). We found that all skeletal and visceral muscles in the abdomen of *T(3;het)*fru^{w12}/*In(3R)*fru^{w27} ($n = 11$), *Df(3R)*fru^{w24}/*In(3R)* fru^{w27} ($n = 50$), and fru^{w12}/fru^{w24} (male, $n = 2$; female, $n = 2$) animals were innervated. However, fewer motorneuronal terminals were present on the lateral muscles of the abdomen. These muscles are arranged as a series of parallel muscle fibers from the lateral edge of the dorsal tergite to the ventral sternite (Figure 4). In wild-type males and females, these muscles are innervated by

TABLE 9

Lethal phase and phenotypes of *fru* mutants

Genotype	*N*	% prepupal (stage P2–P4)	% midpupal (stage P5–P13)	% late pupal (stage P14–P15ii)	% adult with dissection
A. *fru* genotypes with just P4 function[a]					
fru^{w27}/fru^{w24}	65		9	30	61
fru^{w12}/fru^{w27}	95		13	4	80(3[b])
fru^{w12}/fru^{w24}	69	7	62	4	26
$fru^{w12}/P14$	62	44	17	3	36
fru^{w12}/fru^{sat15}	49		6	43	51

Genotype	*N*	% white prepupa (stage P1)	% brown prepupa (stage P2/P3)	% bubble stage (stage P4i/P4II)	% pupal ecdysis (stage P5)
B. *fru* genotypes expressing no *fru* transcripts					
fru^{sat15}/fru^{sat15}	45	100[c]			
fru^{sat15}/fru^{w24}	42		95	5	
$fru^{sat15}/P14$	41		36	63	

	% uneverted discs (at least 1 disc)		% uninflated wing		% 70–90° wing position[d]		% leg joint defects		% duplicated bristles[e]	
Genotype	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*
C. Phenotype of *fru* mutant adults										
$fru^{w12}/P14$ ($M = 14$, $F = 13$)	60	92	85	100	100	100	80	82	100	50
fru^{w12}/fru^{sat15} ($M = 13$, $F = 12$)	31	42	85	92	100	100	69	92	100	100
fru^{w12}/fru^{w24} ($M = 20$, $F = 20$)							100	100	100	100
fru^{w12}/fru^{w27} ($M = 20$, $F = 20$)	15	20	20	20	80	50	30	90	100	100
fru^{w27}/fru^{w24} ($M = 27$, $F = 29$)	0	13	44	30	100	100	92	92	100	96

Animals were collected as white prepupae and aged until they no longer appeared to develop. The stage at which they ceased development was assessed by reference to the metamorphic stages in Bainbridge and Bownes (1981). To simplify presentation of this table some stages were grouped together.

[a] While P3 transcripts are detected by RT-PCR in fru^{w27}, these likely supply little or no *fru* P3 function (see text).

[b] In some genotypes, a small number of animals, in parentheses, emerged on their own (see also Table 3).

[c] fru^{sat15}/fru^{sat15} animals are difficult to stage accurately since the anterior part of the body appears to be at an earlier stage of the metamorphic transition compared to the posterior part of the animal. This anatomical phenotype is more extreme in homozygotes than in heterozygotes with deficiencies for *fru*, such as fru^{w24} or *P14*.

[d] Only wings that were inflated were scored for their position with respect to the body.

[e] Only the anterior scutellar and the sternopleural bristles were obviously duplicated in the thorax.

nerves that have extensive terminal arborizations onto several muscle fibers (Figure 4, A and B). In *fru* mutants that produce just some P3 and P4 or just P4 transcripts, there were fewer branches from the nerve terminal that arborized on these lateral abdominal muscles (Figure 4, C and D; H.-J. Song and B. J. Taylor, unpublished results). In addition, in these *fru* mutants, the innervation of the lateral abdominal muscles was concentrated in the lateral and middle zones, near the dorsal side of the body, and did not penetrate more medial zones of these abdominal muscles as occurs in wild type. These differences in abdominal muscle innervation were not seen in fru^{1} homozygote ($n = 5$) or $Df(3R)Cha^{M5}/In(3R)fru^{w27}$ *trans*-heterozygote ($n = 6$; Figure 4E) mutant flies, suggesting that this synaptic defect is a new *fru* non-sex-specific neuronal phenotype associated with lethal *fru* alleles.

The finding of these defective motorneuronal arborizations in these new fru^{-} mutants led us to ask whether there were any other obvious neuronal phenotypes. We examined postembryonic neurogenesis in $Df(3R)fru^{w24}/In(3R)fru^{w27}$ ($n = 2$), $Df(3R)fru^{w24}/Df(3R)fru^{sat15}$ ($n = 3$), and fru^{1} ($n = 4$) third instar larvae by following the incorporation of BrDU into dividing neuroblasts (Truman and Bate 1989; Taylor and Truman 1992). No difference was detected in the number or pattern of

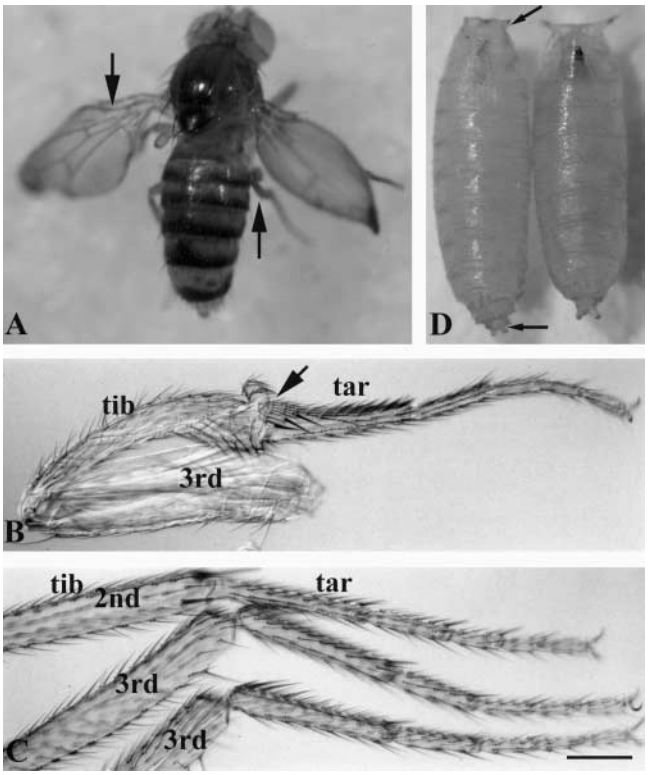

Figure 3.—Anatomical defects in *fru* mutant animals. (A) Photograph of a fru^{w12}/fru^{w27} female, which had been released from the pupal case, showing the partially expanded wings (arrow) and bent tibial segment of the metathoracic leg (lower arrow). (B) Photographs of a metathoracic leg (3rd) from a cuticle preparation of a fru^{w27}/fru^{w24} female with a defective tibial (tib) first tarsal joint (tar; arrow). (C) Photographs of legs from a cuticle preparation of a fru^{1}/fru^{w24} female with normal leg joints. Upper leg is a mesothoracic leg (2nd) and middle and lower legs are left and right metathoracic (3rd) legs. Bar for B and C, 100 μm. (D) Photograph of the ventral side of fru^{sat15}/fru^{w24} (left) and Canton-S (right) 2- to 4-hr prepupae. The fru^{sat15}/fru^{w24} animal has not retracted its head into the typical prepupal form and the anterior (upper arrow) and posterior spiracles (lower arrow) have not fully everted. The mutant prepupa is more elongated than the wild-type prepupa. These *fru* mutants typically die a few hours later after the formation of the posterior bubble.

divisions of larval neuroblasts in the thoracic ganglia or brain in *fru* mutants compared to wild-type males and females (data not shown). In addition, the male-specific abdominal neuroblasts (Truman and Bate 1989; Taylor and Truman 1992) were present and actively dividing in all of the *fru* mutants including $Df(3R)fru^{w24}/Df(3R)fru^{sat15}$ males (data not shown). Since $Df(3R)fru^{w24}/Df(3R)fru^{sat15}$ males and females die just after metamorphosis, the division of the neuroblasts in these mutants shows that the larval program for these neuroblasts is still functioning up until the time of the animals' death. No dividing abdominal neuroblasts were detectable in any *fru* mutant female CNSs from wandering third instar larvae, showing that there is no male transformation of the sex-specific abdominal neuroblasts in *fru* mutant females (data not shown). Although we cannot rule out more subtle defects, the overall division pattern of the postembryonic neuroblasts, which produces the bulk of adult-specific neurons, appears to be normal in *fru* mutants. Thus, of the phenotypes examined, the only neuronal defects detected in the *fru* mutant animals were reduced synaptic arborizations of motorneuronal terminals.

***In situ* expression pattern of *fru* transcripts in *fru* mutants:** The sex-specific expression of *fru* in wild-type males and females is found almost exclusively in neuronal clusters in the brain and ventral nerve cord (Ryner *et al.* 1996; Lee *et al.* 2000). The designations of these clusters on the basis of *in situ* hybridization to *fru* transcripts in sections (Ryner *et al.* 1996) and staining of whole mounts with antibodies to FRU proteins or *in situ* hybridization to *fru* transcripts (Lee *et al.* 2000) differ. Here we give both designations. In the brain, males and females have a number of prominent clusters of labeled neurons in similar locations (Lee *et al.* 2000). We examined several of these for the presence of *fru* transcripts: a cluster distributed in the dorsal posterior protocerebrum, one cluster medial to the optic lobes, three groups in the dorsal anterior protocerebrum, and one near the antennal lobes (Figure 6; Ryner *et al.* 1996; Goodwin *et al.* 2000; M. Foss, G.-H. Lee, E. Reynaud, L. C. Ryner, J. C. Hall, B. S. Baker and B. J. Taylor, unpublished results). We also examined three male-specific groups of neurons in the thoracic-abdominal ganglion; one is located between the pro and mesothoracic neuromeres, the second is in the mesothoracic region, and the third is in the abdominal ganglion (Figure 6; Ryner *et al.* 1996; Goodwin *et al.* 2000; M. Foss, G.-H. Lee, E. Reynaud, L. C. Ryner, J. C. Hall, B. S. Baker and B. J. Taylor, unpublished results).

To determine whether the *fru*-expressing neurons were still present in these *fru* mutant animals we hybridized a riboprobe that detects transcripts initiated at the P1 promoter to sections of pharate adult $Df(3R)fru^{w24}/In(3R)fru^{w27}$ and $T(3;het)fru^{w12}/Df(3R)fru^{w24}$ pupae. This probe should detect transcripts in the mutants even though productive transcripts from the P1 promoter are not made due to lesions within the transcription unit downstream of the region homologous to the probe. We examined sections for the six brain clusters and the three male-specific clusters in the ventral nerve cord mentioned above. Neurons were detected in each of these nine regions of the brain and ventral nerve cord in mutant males and the six regions of the brain in females and these clusters were in locations similar to those of the neuronal groups found in wild-type animals (Figure 5, A and B, and data not shown; Ryner *et al.* 1996; Goodwin *et al.* 2000). Thus, at least some neurons that normally express sex-specific *fru* transcripts are present in these mutant animals, even though no productive sex-specific *fru* transcripts are being made in these cells (Figure 2, B and C). Unlike the expression

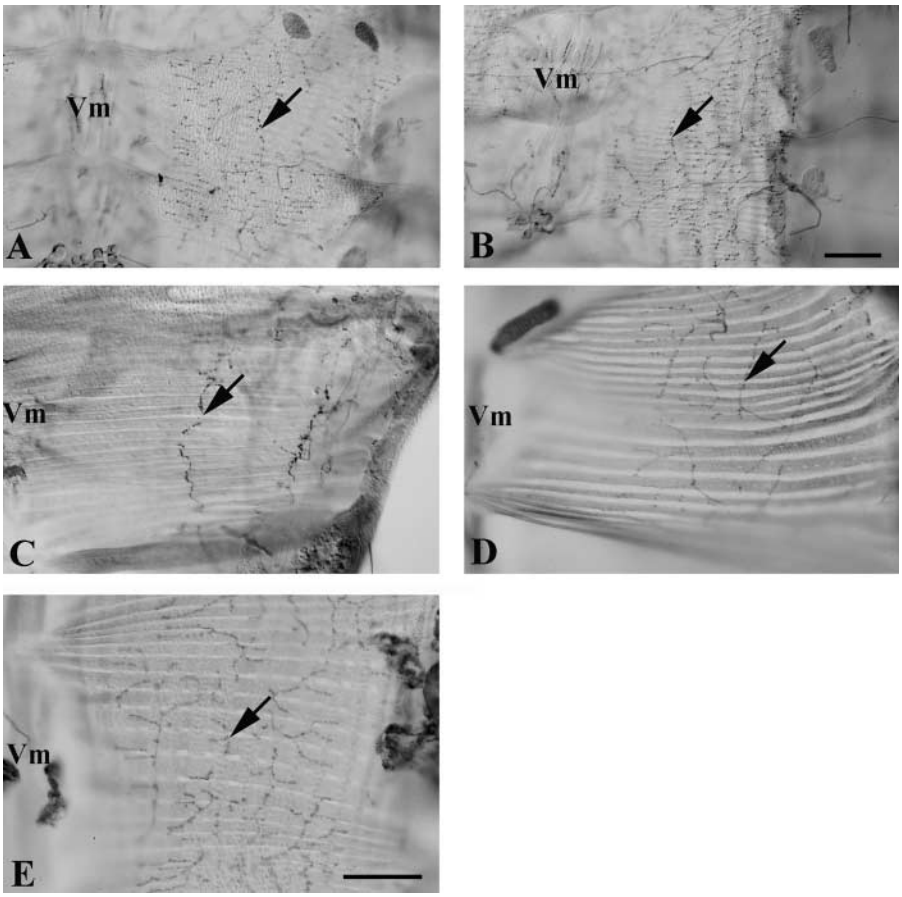

FIGURE 4.—Photomicrograph of lateral A4 abdominal region labeled with anti-synaptotagmin to visualize motor nerve terminals. (A) Canton-S female showing that these muscles are innervated by motorneurons that make highly branched terminal arborizations (arrow). The longitudinal muscles along the ventral midline are indicated by Vm in this and subsequent figures. (B) Canton-S male showing that these muscles are innervated by motorneurons that make highly branched terminal arborizations. Bar for A and B, 50 μm. (C) A fru^{w12}/fru^{w27} female in which the abdominal motorneurons produce fewer lateral terminal arborizations (arrow) as they terminate on the lateral muscles. (D) A fru^{w27}/fru^{w24} male in which the abdominal motorneurons make many fewer terminal branches than wild type. (E) A Cha^{M5}/fru^{w27} female with normal abdominal innervation. Bar for C–E, 50 μm.

pattern in wild-type animals, which shows both nuclear and cytoplasmic signals (RYNER *et al.* 1996), in these *fru* mutants, the label was found only in the nucleus. There was no evidence for cytoplasmic staining, suggesting that the transcripts detected are not transported from the nucleus. The low level of expression in these *fru* mutant animals is likely why this *in situ* hybridization signal was not detected in whole-mount preparations (LEE *et al.* 2000). There were fewer neurons found in these new *fru* mutant animals compared to wild type or to viable *fru* alleles, such as fru^2 (data not shown; GOODWIN *et al.* 2000), suggesting that these transcripts either may be less stable or produced less abundantly or that some *fru*-expressing cells have died or have not differentiated in these *fru* mutants. In homozygous $Df(3R)fru^{sat15}$ third instar larvae labeled with an antisense riboprobe to the P1 promoter, a few labeled neurons were present in the brain, indicating that even in these *fru* mutant larvae that die early during metamorphosis (see above), at least a few *fru*-expressing neurons are present (n =

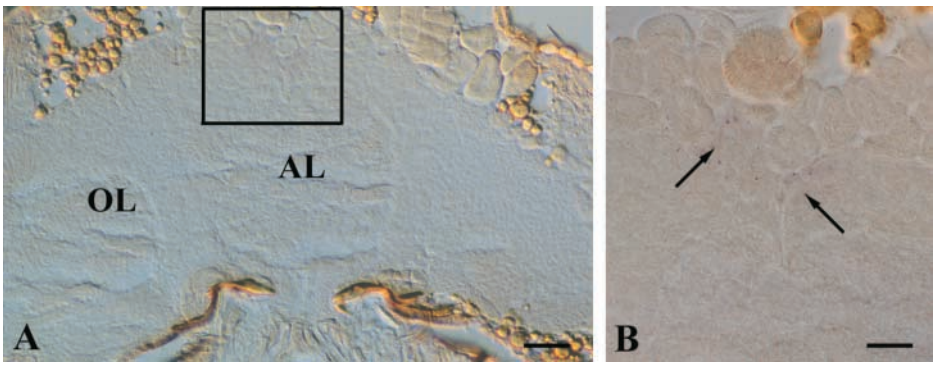

FIGURE 5.—*In situ* hybridization of antisense riboprobes to the P1 promoter (S box in Figure 1B) to sections of fru^{w24}/fru^{w27} mutant male. (A) Low power view of a section through the head at the level of neuronal group 7 (LEE *et al.* 2000; group 6, RYNER *et al.* 1996; see Figure 6) near the antennal lobe. Bar, 60 μm; OL, optic lobe; mcAL, antennal lobe, mechanosensory neuropil. (B) High power view of the boxed region in Figure 5A showing the nuclear label within neurons near the antennal lobe. Arrows point to nuclei that have labeled dots, which are perhaps sites of transcription from the P1 promoter. Bar, 20 μm.

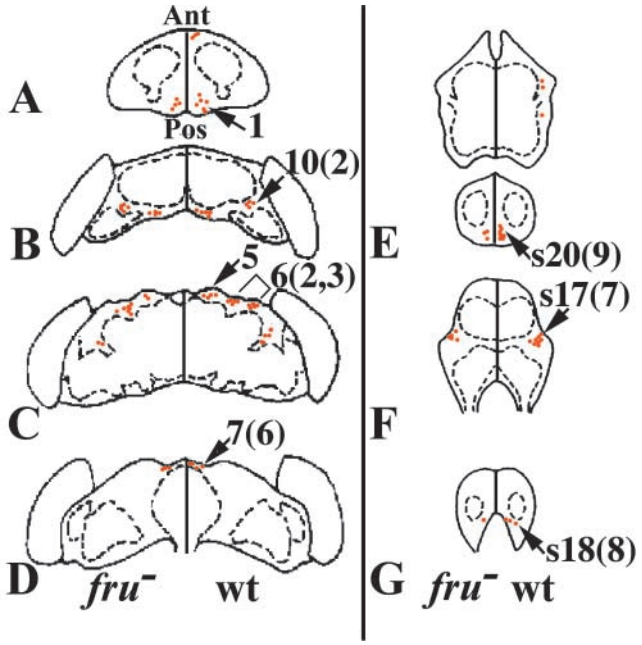

FIGURE 6.—Location of labeled groups of neurons in fru^{w24}/fru^{w27} and wild-type male brain and ventral nerve cord. The sections are selected from a complete series of camera lucida drawings of 20-μm sections through a wild-type animal and represent various horizontal planes through the CNS. These sections were chosen to demonstrate the series of neuronal groups labeled in the CNS of wild-type males by *in situ* hybridization with probes to the P1 promoter (numbers according to LEE *et al.* 2000; numbers in parentheses labeled according to GOODWIN *et al.* 2000; M. FOSS, G.-H. LEE, E. REYNAUD, L. C. RYNER, J. C. HALL, B. S. BAKER and B. J. TAYLOR, unpublished results). In the *fru* mutant male (left), neurons (red dots) are present in regions where the neuronal groups are found in wild-type males (red dots, right) but fewer neurons are labeled. The neuronal clusters were labeled in the brain by their relative dorsal-to-ventral location and in the ventral nerve cord by their anterior-to-posterior location. (A) Dorsal section showing labeled neurons in the dorsal posterior part of the brain (1 in both LEE *et al.* 2000 and GOODWIN *et al.* 2000). (B) Section through the dorsal protocerebrum showing neurons in the lateral group near the optic lobes (10, LEE *et al.* 2000; 2, GOODWIN *et al.* 2000) and neurons from group 1. (C) Section through the protocerebrum showing the location of the three anterior groups (5 and 6, LEE *et al.* 2000; 3–5, GOODWIN *et al.* 2000) distributed along the lateral-to-medial dimension. (D) Ventral section just below the esophagous, which shows the location of the anterior neurons near the mechanosensory antennal lobe (7, LEE *et al.* 2000; 6, GOODWIN *et al.* 2000; Figure 5A). (E) Midway section through the ventral nerve cord showing the location of the abdominal cluster of neurons (a subset of group 20; s20, LEE *et al.* 2000; 9, GOODWIN *et al.* 2000). (F) More ventral section showing the location of the neuronal cluster (a subset of group 17; s17, LEE *et al.* 2000; 7, GOODWIN *et al.* 2000) between the pro- and mesothoracic neuropils. (G) Ventral section showing the location of the mesothoracic neuronal cluster (a subset of group 18, LEE *et al.* 2000; 8, GOODWIN *et al.* 2000).

6; data not shown). In addition, these findings suggest that *fru*-expressing neurons, at least in the major *fru*-expressing clusters of neurons, do not require the production of intact sex-specific *fru* transcripts for survival at least up to the pharate adult stage of development.

In contrast to the signal detected by RT-PCR techniques (Figure 2), essentially no signal was found by *in situ* hybridization using riboprobes generated from the common protein-coding region in $Df(3R)fru^{w24}/In(3R)fru^{w27}$ (male, $n = 3$, female, $n = 3$, pharate adult; male, $n = 2$, female, $n = 1$, midpupal stage) and $T(3;het)fru^{w12}/In(3R)fru^{w27}$ (male, $n = 3$, female, $n = 3$, pharate adult). The *in situs* to these two genotypes were not distinguishable from those to $Df(3R)fru^{w24}/Df(3R)fru^{sat15}$ (third larval instar, male, $n = 3$, female, $n = 3$; data not shown), which is deleted for the common coding region. It may be that the expression of transcripts detected by RT-PCR in $Df(3R)fru^{w24}/In(3R)fru^{w27}$ and $T(3;het)fru^{w12}/In(3R)fru^{w27}$ is below the level of detection by *in situ* hybridization or that they are expressed in other tissues (see DISCUSSION).

DISCUSSION

The initial molecular characterizations of *fru* (RYNER *et al.* 1996; HEINRICHS *et al.* 1998; GOODWIN *et al.* 2000) showed that it produced a complex array of products through the use of at least three promoters and alternative splicing. We undertook the isolation and characterization of additional *fru* alleles with two major goals in mind: first, to gain insight into the relationship between *fru*'s various functions and the complex array of products produced by *fru*; second, to characterize in more detail the phenotypic effects of *fru* mutations to better understand the nature of *fru*'s wild-type functions. The mutations reported here have already contributed significantly to our understanding of *fru*. Thus a preliminary characterization of several of the new alleles reported here (RYNER *et al.* 1996) showed that *fru* encoded a sex-nonspecific vital function. In addition, the finding that *fru* transcripts could still be detected in genotypes generated with these mutations that lacked all previously known *fru* promoters (P1–3) led to the identification of the P4 promoter (L. C. RYNER, S. F. GOODWIN, T. CARLO, M. FOSS, J. C. HALL, B. J. TAYLOR and B. S. BAKER, unpublished results).

On the relationships between *fru*'s products and functions: We proposed (RYNER *et al.* 1996) that *fru*'s role in controlling male sexual behavior was through the products of the distal (P1) *fru* promoter. Briefly, the reasoning was as follows. Genetic analysis (TAYLOR 1992; TAYLOR *et al.* 1994) had shown that while all aspects of male somatic sexual differentiation were controlled by the *transformer* (*tra*) and *transformer-2* (*tra-2*) sex determination genes, *doublesex* (*dsx*), the only known sex determination regulatory gene below *tra* and *tra-2* in the sex determination hierarchy, did not control two aspects of male sexual differentiation (the MOL and male sexual behavior). These results indicated that there was a previously unrecognized branch of the sex determination

hierarchy below *tra* and *tra-2* and led to the molecular search for other genes that were direct targets of *tra* and *tra-2*. That search identified *fru* as a direct target of *tra* and *tra-2* in that the transcripts from the P1 *fru* promoter were sex-specifically alternatively spliced under the control of the TRA and TRA-2 proteins (Ryner *et al.* 1996; Heinrichs *et al.* 1998). That *fru* function was known to be required for some aspects of male sexual behavior and the formation of the MOL (Gill 1963; Hall 1978; Gailey and Hall 1989; Gailey *et al.* 1991) and that the *fru* P1 transcripts were expressed in regions of the CNS previously implicated in certain aspects of male sexual behavior (Ryner *et al.* 1996) made a strong case for the products of the P1 *fru* promoter functioning to control aspects of sexual differentiation in the CNS needed for male sexual behavior and the MOL formation. However compelling that case may seem, it falls short of establishing that the products of the *fru* P1 promoter have the proposed functions, since there was no demonstration that mutations that impair P1 *fru* function alone had the expected phenotypes. Recent data consistent with the proposed function of P1 *fru* products have come from the following findings: (1) the fru^{1} allele, which affects male sexual behavior, is an inversion broken 5′ to the P1 promoter and alters the spatial pattern of P1 expression in the CNS (and based on its location probably does not affect expression from the other *fru* promoters; Goodwin *et al.* 2000); and (2) the fru^{3} and fru^{4} alleles, which have strong effects on male sexual behavior (Villella *et al.* 1997), result in a substantial reduction in the amount of normal P1 and P2 (but not P3 and P4)-derived transcripts (Goodwin *et al.* 2000).

Further support for the proposed roles of the P1 *fru* transcripts is provided by various genotypes involving the new *fru* alleles reported here. Our findings that males, which express P3 and P4 transcripts, but no P1 or P2 transcripts [*i.e.*, $Df(3R)fru^{4\text{-}40}/Df(3R)P14$, $Df(3R)fru^{4\text{-}40}/T(3;Y)fru^{w9}$, $Df(3R)fru^{4\text{-}40}/T(3;het)fru^{w12}$, $Df(3R)fru^{4\text{-}40}/In(3R)fru^{w27}$, and $Df(3R)fru^{4\text{-}40}/Df(3R)fru^{sat15}$], are sterile and exhibit little or no male-specific reproductive behaviors in single-pair tests strongly support the hypothesis that the products encoded by *fru*'s P1 (and possibly P2) transcripts control male sexual behavior. Flies of some of the above genotypes show reduced general activity and thus one might argue that this contributed to their reduced sexual behavior. However, as quantified in results, there was in general only a weak correlation between the scores on courtship tests and the test of general activity. Moreover, $Df(3R)fru^{4\text{-}40}/Df(3R)P14$ males had wild-type levels of general activity and $Df(3R)fru^{4\text{-}40}/T(3;Y)fru^{w9}$ and $Df(3R)fru^{4\text{-}40}/Df(3R)fru^{sat15}$ males had moderate levels of activity, indicating that for these genotypes the absence of male sexual behavior is unrelated to any general behavioral deficit.

That males having P1-encoded functions either lacking or seriously impaired show as severe decrements in their sexual behavior as males lacking both P1- and P2-encoded functions suggests that *fru*'s role in sexual behavior may be entirely attributed to its P1-encoded functions. Males lacking or severely subnormal for P1 functions are represented by $Df(3R)Cha^{M5}$ heterozygous with $T93;Y)fru^{w9}$, $T(3;het)fru^{w12}$, $In(3R)fru^{w27}$, or $Df(3R)fru^{sat15}$. In single-pair tests of courtship behavior, males of all these genotypes show essentially no sexual behavior. Moreover, all four types of males showed either normal or moderate levels of general activity. Since a genotype that lacks only P2-encoded functions is not available, we cannot exclude the possibility that P2-encoded functions are also important for male sexual behavior. However, all of the data currently available can be explained by the proposal that *fru*'s P1-encoded functions are responsible for *fru*'s role in male sexual behavior.

The new *fru* genotypes we analyzed also provide some insight into which *fru* products are necessary for *fru*'s vital function. With respect to whether P2 encodes a vital function, two aberrations, $Df(3R)fru^{4\text{-}40}$ and $In(3R)fru^{w27}$, both of which lack P1 and P2 transcripts and produce P3 and P4 transcripts, gave completely discordant results when used in complementation tests with other *fru* alleles that lack *fru*'s vital function. Thus $Df(3R)fru^{4\text{-}40}$ over $Df(3R)fru^{sat15}$ or $Df(3R)P14$ is fully viable (Table 3C), whereas $In(3R)fru^{w27}$ over $Df(3R)fru^{sat15}$, $Df(3R)Cha^{M7}$, $Df(3R)fru^{w24}$, $T(3;het)fru^{w12}$, or $T(3;Y)fru^{w9}$ is lethal (Table 3D). We believe that this disparity is due to $In(3R)fru^{w27}$ being defective in more than just *fru*'s P1 and P2 functions. Our reasoning is as follows. First, the $In(3R)fru^{w27}$ chromosome is a complex rearrangement having four cytologically detectable breakpoints (Table 1), one of which is in *fru*. $In(3R)fru^{w27}$ has two molecularly identified lesions in *fru,* one lesion being in the DNA of the fru^{2} *P* element on which $In(3R)fru^{w27}$ was induced (which is located between P3 and P4; Figure 1B) and the second lesion, presumably the cytologically detectable one, being between P2 and P3 (Figure 1B) and preventing transcripts from going from P1 and P2 but not from P3 and P4 to the common coding region. Second, in viability tests, as well as all the other phenotypic tests we carried out, $In(3R)fru^{w27}$ behaves like $T(3;Y)fru^{w9}$ and $T(3;het)fru^{w12}$, which lack P1, P2, and P3 function. While $In(3R)fru^{w27}$ has P3 transcripts detected by RT-PCR, its phenotypic similarity to $T(3;Y)fru^{w9}$ and $T(3;het)fru^{w12}$ suggests it does not have P3 function. These observations can be reconciled if $In(3R)fru^{w27}$ either does not produce P3-derived proteins or expresses them in aberrant temporal or spatial patterns. With respect to the latter possibility note that $In(3R)fru^{w27}$ is associated with an inversion broken in *fru,* which is likely between P2 and P3, and would thus juxtapose new sequences upstream of P3. These new sequences may either lead to abnormal expression of P3 or introduce an ectopic promoter. If this reasoning with respect to $In(3R)fru^{w27}$ is correct, and the $Df(3R)fru^{4\text{-}40}$ results are reflective of the loss of both P1 and P2 functions, then P2-derived products, like those

derived from P1, are not needed for viability. Thus *fru*'s vital functions must be carried out by P3- and/or P4-derived products. That P3-encoded functions are required for viability is further suggested by the lethality of a number of mutant combinations that lack P1, P2, and P3 functions (Table 3) but retain P4 function. We could not determine whether the products of the P4 *fru* promoter encode a vital function, since there is no genotype that lacks P4 function but has P3 function.

In summary, our studies implicate the products of the P1 *fru* promoter as being responsible for *fru*'s control of male sexual behavior and the products of the P3 *fru* promoter for carrying out *fru*'s vital function. The functions of the products of the P2 and P4 promoters are currently unclear. However, it needs to be noted that we do not have genotypes that individually remove the functions encoded by P2-, P3-, or P4-derived transcripts. Thus our conclusions as to the functions of P1- and P3-derived transcripts are the simplest ones compatible with our data, but we cannot exclude the possibilities that P2-, P3-, or P4-derived products may play some role in male sexual behavior or that P4-encoded products may also carry out a vital function. Moreover, while our findings that flies lacking P1 and P2 promoter-derived products are viable whereas flies lacking P1-, P2-, and P3-derived products are lethal are most simply compatible with the proposal that P3 products carry out a vital function, these data do not preclude some models in which there is redundancy between the P1, P2, and P3 products in providing *fru*'s vital function.

***fru*'s role in male sexual behavior:** From a detailed analysis of the new *fru* alleles we showed that wild-type *fru* function is necessary for the production of nearly all aspects of male sexual behavior. *fru* mutant males lacking P1-encoded products no longer performed any courtship behavior with either single male or female partners in standard courtship tests (Table 5). The observed failure of males of various *fru* genotypes to court might reflect a specific defect in sexual behavior, or alternatively it might be a by-product of a general reduction in activity. Although males of several *fru* genotypes did have reduced levels of short-term activity, regression analysis showed that, overall, there was only a poor correlation between the level of activity and male courtship behavior; this indicates that low activity by these males could at most account for only a small part of the variation in male courtship behavior. Moreover, the reduction in courtship in single-pair tests occurred in some males that had moderate to high levels of activity [*e.g.*, $Df(3R)fru^{4\text{-}40}/Df(3R)P14$ or $Df(3R)fru^{4\text{-}40}/Df(3R)fru^{sat15}$]. Further evidence indicating that reduced courtship with a single partner is a specific defect is shown by a number of *fru* genotypes in which males did not court a male or female partner, for example, $T(3;Y)fru^{w9}/fru^{3}$, but did engage in male-male group courtship as measured by the ChI. This dichotomy in CI *vs.* ChI shows that males were capable of generating at least some courtship behaviors in one situation but did not express that similar behavior in a different setting. Thus, the elimination of male courtship to both males and females in a subset of *fru* mutant genotypes reflects a sex-specific courtship effect of the loss of fru^{+} function.

There are two ways these results could be interpreted in terms of the wild-type function of *fru*. First, since male courtship is a dependent action pattern, with the occurrence of one step in the courtship sequence generally requiring the completion of preceding steps, these results could simply mean that these *fru* males are blocked in some way prior to the very earliest steps of courtship. Alternatively these results could mean that fru^{+} function is necessary for each step of courtship. The data support the latter alternative. Using several hypomorphic alleles, previous studies showed that *fru* mutant males courted males and females but were blocked at certain individual steps occurring during courtship. For example, fru^{3} and fru^{4} males are blocked in the middle of the courtship behavioral sequence such that although they court they do not vibrate their wings to generate the courtship song and do not attempt copulation (Villella *et al.* 1997). By comparison, fru^{1} males are blocked only in the penultimate step of attempted copulation (Hall 1978; Gailey and Hall 1989; Gailey *et al.* 1991; Villella *et al.* 1997). These studies provided strong evidence that male-specific fru^{+} function is involved with the specific neuronal circuitry required for the wing vibration needed for courtship song and the abdominal bending for copulation (Ryner *et al.* 1996). Various heteroallelic combinations we tested also show similar defects at intermediate to late stages of courtship. Thus many of the combinations of new *fru* alleles with fru^{1} or fru^{2} showed significant levels of courtship activity as measured by either the CI or the WEI yet were sterile or had greatly reduced fertility due to failures to copulate. Similarly, a number of the *fru* genotypes we tested failed to produce a courtship song during the reduced period that they were extending their wings. Taken together, these findings suggest that *fru* functions to establish the potential for essentially all aspects of male sexual behavior.

The exact molecular role that the P1 products play in controlling male-specific behaviors is not known, but they are likely to affect the development and differentiation of particular male-specific neurons and neuronal circuits. Of the clusters of P1-expressing neurons that were identified, we focused on nine different clusters of P1-expressing neurons that were identified by *in situ* hybridization in late stage pharate male brains and are prominent in sectioned preparations (Ryner *et al.* 1996; Goodwin *et al.* 2000). Males of a genotype [$T(3;het)\text{-}fru^{w12}/In(3R)fru^{w27}$] that did not produce any courtship behavior appear to retain many or most of these neurons in the CNS even though these animals are not producing any functional P1 or P2 transcripts. This finding strongly suggests that the primary role of the P1 tran-

scripts is to direct sex-specific differentiation and not direct the production or facilitate the survival of these neurons.

On single-pair *vs.* group courtship: One aspect of the courtship phenotypes of the *fru* mutant combinations that requires special consideration is what might appear to be somewhat discordant results between single-pair courtship tests (both male-male and male-female) and the male-male group courtship (chaining) test. Of the 10 genotypes that we tested that lacked P1 function (Table 5, B and C), all displayed little or no courtship in single-pair tests, but 6 of these genotypes displayed significant levels of male-male group courtship. In addition, of the 15 genotypes we tested in which one of the new alleles was heterozygous with either fru^2, fru^3, or fru^4, 12 genotypes displayed little or no courtship in single-pair tests (the exceptions all being combinations involving $Df(3R)fru^{4\text{-}40}$), yet 13 of these genotypes had significant, often substantial, levels of male-male group courtship. In thinking about these data it is important to recall that although single-pair male-female courtship, single-pair male-male courtship, and male-male group courtship are all referred to as courtship, male-male group courtship is a mutant phenotype, whereas single-pair male-female courtship is the wild-type phenotype in *D. melanogaster*. Mature males courting each other in pairs essentially never occurs in a sustained manner in this species, so single-pair male-male courtship might also be viewed as a "mutant" phenotype. Thus one might expect *a priori* that there would not be parallel dependencies of these three different "courtship" phenotypes on *fru* function. In that regard it is worth recalling that the levels of courtship as measured by the CIs of males of a given genotype with single males or single females are highly correlated. Strikingly, a similar linear regression analysis of the mean $CI_{m\text{-}m}$ and the ChI across all *fru* genotypes showed a similarly strong positive correlation. These high degrees of concordance suggest that these three behavioral assays are measuring behaviors that are largely equivalent in their *fru* dependency. The difference between wild type and any of the *fru* mutants that have been tested to date would be that in the *fru* mutant situations males as well as females would be seen as appropriate partners for whatever level of courtship males of a particular genotype can attain.

As was just noted for a number of genotypes males showed little or no courtship in single-pair tests but were able to carry out the very first steps of courtship (orientation and following), but not later steps, of courtship in group tests. These differences within a genotype are likely related to the different ways single-pair and group tests are done. In particular, male-male group tests are carried out by placing a group of males together for 3–4 days prior to testing, whereas males used in single-pair courtship tests were stored individually until tested. The richer environment experienced by males used in male-male group courtship tests (more stimulation through dynamic interactions between animals and a longer time for association between animals) is likely the reason for the different levels of courtship seen in single-pair and group tests. The preceding interpretation is suggested by the prior observation that fru^2, fru^3, and fru^4 homozygous males chained little, if at all, when initially placed together in groups, but that the level of chaining increased over the next couple of days (Villella *et al.* 1997). The finding that *fru* mutant animals perform some courtship steps under group conditions also shows that the appropriate parts of the brain for at least the very initial steps of courtship are still present and can be activated in these genotypes but that the conditions of the test for pairwise courtship do not lead to the activation of these same centers and the expression of orientation and following.

Another striking aspect of these data is that 6 of the 10 genotypes that completely lack P1 promoter-encoded functions showed significant levels of male-male group courtship. The finding that 4 of these 10 genotypes [$T(3;het)fru^{w12}/Df(3R)Cha^{M5}$, $T(3;het)fru^{w12}/Df(3R)fru^{4\text{-}40}$, $T(3;het)fru^{w12}/In(3R)fru^{w27}$, and $Df(3R)fru^{4\text{-}40}/Df(3R)P14$], as well as the previously characterized $Df(3R)Cha^{M5}/Df(3R)P14$ combination (Villella *et al.* 1997), showed no courtship in either single pairs or as groups of males is consistent with the conclusion that it is the products of the P1 *fru* promoter that establish the potential for male sexual behavior. However, the results from the 6 genotypes that show significant male-male group courtship suggest that other factors are involved that we do not understand. There does not seem to be an obvious genetic explanation for these data, since genotypes that should be identical in terms of their arrays of *fru* products do not have equivalent phenotypes. For example, in $Df(3R)Cha^{M5}/Df(3R)P14$ males all courtship behavior is abolished (Villella *et al.* 1997), while $Df(3R)Cha^{M5}/Df(3R)fru^{sat15}$ males showed no courtship behavior in single-pair tests but had a ChI = 36. Similarly, $T(3;Y)fru^{w9}$, $T(3;het)fru^{w12}$, and $In(3R)fru^{w27}$ over $Df(3R)Cha^{M5}$ had ChI's of 19, 5, and 36, respectively, yet our data suggest these should be equivalent in terms of *fru* function. The origin of these differences is unclear.

***fru*'s role in MOL development:** We suggested that the products of the P1 *fru* promoter are responsible for both MOL development and male sexual behavior. An obvious question is whether this *fru*-regulated male-specific muscle has any connection with *fru*-regulated male-specific courtship behavior. To date, the physiological function of the MOL is unknown. It has been demonstrated that the MOL is not necessary for copulation to occur in this species (Gailey *et al.* 1991), and, in fact, the males of most Drosophila species do not even develop a MOL (Gailey *et al.* 1997).

Given the data indicating that the P1-derived male-specific FRU proteins govern MOL development, the recent report (Usui-Aoki *et al.* 2000) that expression of a *fru* cDNA construct encoding a protein lacking the

101-amino-acid male-specific N terminus could partially rescue the MOL phenotype in fru^{sat} homozygous males and lead to MOL development in wild-type females was surprising. The fact that a FRU protein can induce MOL development in an otherwise wild-type female provides striking evidence that FRU function is sufficient to trigger MOL development. With respect to the question of which class of FRU proteins controls MOL development in wild type, we note the following. The FRU protein shown by Usui-Aoki *et al.* (2000) to be capable of producing a MOL is identical to a P1-derived FRU protein, except for the absence of the male-specific 101-amino-acid N terminus. It would not be surprising if there were some overlap in the functionality of these two proteins. The heat-shock and GAL4 expression systems used by Usui-Aoki *et al.* (2000) are likely to produce FRU protein at significantly higher levels than that protein is present in wild type. Thus the production of MOLs in their experiments may be being brought about by the overexpression of a protein whose function partially overlaps that of P1-encoded proteins. Alternatively, it could be that these proteins are functionally fully synonymous, and these functions are biologically determined by the locations and levels at which they are expressed.

***fru*'s vital function:** Our studies of the phenotypes of lethal *fru* mutant combinations provided insights into the nature of *fru*'s essential function. Genotypes that lack P3 (and P1 and P2) promoter function [$T(3;het)fru^{w12}/Df(3R)fru^{sat15}$, $T(3;het)fru^{w12}/Df(3R)fru^{w24}$, $T(3;het)fru^{w12}/In(3R)fru^{w27}$, and $T(3;het)fru^{w12}/Df(3R)P14$] have lethal phases in the mid- to late pupal period, but in all cases a substantial fraction of these individuals are viable if assisted in emerging from the pupal case. Thus the vital function encoded by P3 is only essential very late in development.

P3-encoded functions appear to be necessary for the differentiation of imaginal-disc derivatives, such as legs and wings. The rare survivors with *fru* lethal genotypes, such as $T(3;het)fru^{w12}/In(3R)fru^{w27}$, showed defects in their external morphology and had specific sensory bristle organs duplicated. Moreover, imaginal discs frequently failed to evert in adult escapers of genotypes that lacked P3 function. These observations suggest that there is a role for *fru* in imaginal disc development or differentiation. The gross morphological defects produced in adult derivatives of imaginal discs make it likely that the nearly lethal phenotype of *fru* genotypes lacking P3 function arises from these defects.

In addition, many of the adult escapers that lacked P3 function had neuroanatomical defects in the motor innervation to the abdominal muscles. These defects in the motorterminals may explain part of the inability of many of these *fru* mutant genotypes to successfully eclose from the pupal case. All abdominal muscles appear to be innervated, suggesting that the defects in these *fru* mutants are not due to abnormalities in neuronal pathfinding but rather some feature of synaptogenesis once the motorneuronal axons reached their target muscles. Changes in the branching of motorneurons at the neuromuscular junction in larval Drosophila were described for various mutants that affect neuronal excitability. The number and size of motorneuronal terminals are increased on larval muscles in *Shaker* and *Shaker ether-a-go-go* mutant animals, which show increased neuronal excitability, whereas terminals are reduced in number and size in *no action potential* and *paralytic* mutants with reduced excitability (Budnik *et al.* 1990; Jia *et al.* 1993; reviewed in Keshishian *et al.* 1996). In other respects the development of the nervous system of *fru* mutant genotypes appeared normal. We did not find any obvious change in the number or patterns of divisions of postembryonic neuroblasts in either a null *fru* genotype [$Df(3R)fru^{w24}/DF(3R)fru^{sat15}$] or a genotype lacking P1, P2, and P3 functions [$Df(3R)fru^{w24}/In(3R)fru^{w27}$], suggesting that the normal complement of adult-specific neurons is likely produced in the complete absence of *fru* function. The apparently normal level of postembryonic divisions of neuroblasts in larval CNSs of $Df(3R)fru^{sat15}/Df(3R)fru^{w24}$, which are destined to die within a day, makes it unlikely that the loss of adult neurons is responsible for the death of these animals.

While there is currently not a *fru* genotype that is null for all *fru* functions and wild type for neighboring genes, it was possible to place a limit on how extreme a completely null *fru* genotype might be by examining the lethal phase of mutant combinations [$Df(3R)fru^{sat15}/Df(3R)fru^{sat15}$ and $Df(3R)fru^{sat15}/Df(3R)fru^{w24}$] that were null for *fru* as well as a small number of adjacent genes. The development of these individuals is arrested in early pupal development, around the time of pupal ecdysis (Table 9). The morphology of the pupal case in these two $Df(3R)fru^{sat15}$ genotypes is similar to the phenotypes of animals mutant for the ecdysone receptor and the *crooked legs* gene (Bender *et al.* 1997; D'Avino and Thummel 1998). However, the above *fru* genotypes lack not only all *fru* function but also the function of at least four, and possibly six, neighboring genes. Thus it is not clear if the earlier lethal phase caused by these two genotypes—as compared to *fru* genotypes that are lacking just P1, P2, and P3 function—is a consequence of the absence of P4 function or the absence of one of the neighboring genes. In any case, these two null genotypes establish that a complete absence of *fru* function does not result in a lethal phase prior to the beginning of the pupal period.

In summary, our results establish that *fru* functions sex-specifically in the sex-determination regulatory hierarchy to control male sexual behavior and sex-nonspecifically to control the development of imaginal discs and motorneuronal synapses during development. Perhaps most importantly these results show that just the absence of the products of the P1 *fru* promoter results in the loss of all normal male sexual behavior and thus provide strong support for the proposal (Ryner *et al.*

1996) that these proteins function to establish the potential for nearly all components of a Drosophila male's sexual behavior.

We thank R. Levis for $p^{p}[w\,A]$ DNA and H. Bellen for synaptotagmin antibody.

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.

Bainbridge, S. P., and M. Bownes, 1981 Staging the metamorphosis of *Drosophila melanogaster*. J. Embryol. Exp. Morphol. **66:** 57–80.

Bender, M., F. B. Imam, W. S. Talbot, B. Ganetsky and D. S. Hogness, 1997 Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell **91:** 777–788.

Bernstein, A. S., E. K. Neumann and J. C. Hall, 1992 Temporal analysis of tone pulses within the courtship songs of two sibling Drosophila species, their interspecific hybrid, and behavioral mutants of *D. melanogaster*. J. Insect Behav. **5:** 15–36.

Budnik, V., Y. Zhong and C.-F. Wu, 1990 Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. J. Neurosci. **10:** 3754–3768.

Castrillon, D. H., P. Gonzy, 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.

D'Avino, P. P., and C. S. Thummel, 1998 *crooked legs* encodes a family of zinc finger proteins required for leg morphogenesis and ecdysone-regulated gene expression during Drosophila metamorphosis. Development **125:** 1733–1745.

Finley, K. D., B. J. Taylor, M. Milstein and M. McKeown, 1997 *dissatisfaction*, a gene involved in sex-specific behavior and neural development of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **94:** 913–918.

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.

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.

Gailey, D. A., S. Ohshima, S. Santiago, J. Montez, A. Arellano *et al.*, 1997 The muscle of Lawrence in Drosophila: a case of repeated evolutionary loss. Proc. Natl. Acad. Sci. USA **94:** 4543–4547.

Gill, K. S., 1963 A mutation causing abnormal courtship and mating behavior in males of Drosophila melanogaster (abstr.) Am. Zool. **3:** 507.

Goodwin, S. F., B. J. Taylor, A. Villella, M. Foss, L. C. Ryner *et al.*, 2000 Aberrant splicing and altered spatial expression patterns in fruitless mutants of *Drosophila melanogaster*. Genetics **154:** 725–745.

Hall, J. C., 1978 Courtship among males due to a male-sterile mutation in Drosophila melanogaster. Behav. Genet. **8:** 125–141.

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

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.

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. Fusjitani, 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.

Jia, X.-X., M. Gorczyca and V. Budnik, 1993 Ultrastructure of neuromuscular junctions in *Drosophila*: comparison of wild type and mutants with increased excitability. J. Neurobiol. **24:** 1025–1044.

Keshishian, H., K. Brodie, A. Chiba and M. Bate, 1996 The *Drosophila* neuromuscular junction: a model system for studying synaptic development and function. Annu. Rev. Neurosci. **19:** 545–575.

Kulkarni, S. J., and J. C. Hall, 1987 Behavioral and cytogenetic analysis of the *cacophony* courtship song mutant and interacting genetic variants in *Drosophila melanogaster*. Genetics **115:** 461–475.

Lee, G., M. Foss, S. F. Goodwin, T. Carlo, B. J. Taylor *et al.*, 2000 Spatial, temporal, and sexually dimorphic expression patterns of the fruitless gene in the Drosophila central nervous system. J. Neurobiol. **43:** 404–426.

Lee, G., A. Villella, B. J. Taylor and J. C. Hall, 2001 New reproductive anomalies in *fruitless*-mutant *Drosophila* males: extreme lengthening of mating durations and infertility associated with defective serotonergic innervation of reproductive organs. J. Neurobiol. **147:** 121–149.

Levis, R., T. T. Hazelrigg and G. M. Rubin, 1985 Separable cis-acting control elements for expression of the white gene of Drosophila. EMBO J. **4:** 3489–3499.

Lindsley, D. L., and G. G. Zimm, 1992 The genome of *Drosophila melanogaster*. Academic Press, San Diego.

Littleton, J. T., H. J. Bellen and M. S. Perin, 1993 Expression of synaptotagmin in Drosophila reveals transport and localization of synaptic vesicles to the synapse. Development **118:** 1077–1088.

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.

Robertson, H. M., C. R. Preston, R. W. Philis, D. Johnson-Schiltz, W. K. Benz *et al.*, 1988 A stable genomic source of *P*-element transposase in *Drosophila melanogaster*. Genetics **118:** 461–470.

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.

Sokal, R. R., and F. J. Rohlf, 1995 *Biometry*, Ed. 3. W. H. Freeman, New York.

Szabad, J., 1978 Quick preparation of Drosophila for microscopic analysis. Dros. Inf. Serv. **53:** 1215.

Taylor, B. J., 1992 Differentiation of a male-specific muscle in *Drosophila melanogaster* does not require the sex-determining genes *doublesex* or *intersex*. Genetics **132:** 179–191.

Taylor, B. J., and L. Knittel, 1995 Sex-specific differentiation of a male-specific abdominal muscle, the Muscle of Lawrence, is abnormal in hydroxyurea-treated and in *fruitless* male flies. Development **121:** 3079–3088.

Taylor, B. J., and J. W. Truman, 1992 Commitment of abdominal neuroblasts in Drosophila to a male or female fate is dependent on genes of the sex-determining hierarchy. Development **121:** 3079–3088.

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. **14:** 411–440.

Truman, J. W., and M. Bate, 1989 Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. Dev. Biol. **125:** 145–157.

Usui-Aoki, K., H. Ito, K. Ui-Tei, K. Takahashi, T. Lukacsovich *et al.*, 2000 Formation of the male-specific muscle in female Drosophila by ectopic *fruitless* expression. Nat. Cell Biol. **2:** 500–506.

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.

Wheeler, D. A., W. L. Fields and J. C. Hall, 1988 Spectral analysis of Drosophila courtship songs: *D. melanogaster*, *D. simulans* and their interspecific hybrid. Behav. Genet. **18:** 673–703.

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.

Communicating editor: R. S. Hawley