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Genetics

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

(homosexual courtship/muscle of Lawrence/transformer/mating behavior)

HIROKI ITO, KAZUKO FUJITANI, KAZUE USUI, KEIKO SHIMIZU-NISHIKAWA*, SHOJI TANAKA, AND DAISUKE YAMAMOTO†

Yamamoto Behavior Genes Project, Exploratory Research for Advanced Technology (ERATO), Research Development Corporation of Japan, and Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan

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ABSTRACT **We have isolated a new *Drosophila* mutant, satori (*sat*), the males of which do not court or copulate with female flies. The *sat* mutation comaps with fruitless (*fru*) at 91B and does not rescue the bisexual phenotype of *fru*, indicating that *sat* is allelic to *fru* (fru^{sat}). The fru^{sat} adult males lack a male-specific muscle, the muscle of Lawrence, as do adult males with other *fru* alleles. Molecular cloning and analyses of the genomic and complementary DNAs indicated that transcription of the *fru* locus yields several different transcripts. The sequence of *fru* cDNA clones revealed a long open reading frame that potentially encodes a putative transcription regulator with a BTB domain and two zinc finger motifs. In the 5′ noncoding region, three putative transformer binding sites were identified in the female transcript but not in male transcripts. The *fru* gene is expressed in a population of brain cells, including those in the antennal lobe, that have been suggested to be involved in determination of male sexual orientation. We suggest that *fru* functions downstream of *tra* in the sex-determination cascade in some neural cells and that inappropriate sexual development of these cells in the *fru* mutants results in altered sexual orientation of the fly.**

A powerful approach to investigating the biological basis of sexual orientation is to use animal models that allow experimental manipulation of complex behavior. *Drosophila melanogaster* provides the combination of identified neurons of known projection, the applicability of classic genetic theory, and the potential for advanced molecular biological analysis, making it an excellent organism for investigating higher neural functions such as sexual orientation (1–4). We have screened about 2000 fly lines with single P-element insertions for altered sexual behavior, yielding a mutant named satori (*sat*; nirvana in Japanese), the males of which do not court or copulate with females. Instead, *sat* males exhibit homosexual courtship. We report here that *sat* is allelic to fruitless (*fru*) whose dysfunction is known to lead to "bisexual" behavior (5–7) and loss of a male-specific muscle, the muscle of Lawrence (MOL) (8, 9). We further show that fru^{sat} is a likely transformer (*tra*) target, encodes a putative transcription factor with a BTB domain (10) and two zinc finger motifs, and is expressed in a subset of cells in the central nervous system.

MATERIALS AND METHODS

Mutagenesis, Mutant Screening, and Phenotype Analysis. The jump-start method was used for mutagenesis with the PlwB element as a mutator and the P ($ry^+\Delta 2\text{-}3$) transposon as a jump starter. All flies used in the mutagenesis had a white$^-$ (w^-) background, whereas the PlwB element carried a copy of w^+, allowing us to recover chromosomes with PlwB insertions by selecting individuals with nonwhite eye color. After establishing fly lines with new insertions, homozygous virgin males and females were collected at eclosion, placed singly in food vials, and aged for 3 days. For behavior screening, single male and female pairs were introduced into disposable plastic syringes (volume, 1 cm^3). At least 10 pairs per strain were visually observed for 1 h, and the time to copulation, duration of copulation, and percentage of pairs copulating were recorded. In this screen, we isolated seven mutations, *sat*, croaker (*cro*) (11), fickle, okina, spinster (7), chaste, and lingerer (see ref. 4 for further details of these mutations). By introducing the P ($ry^+\Delta 2\text{-}3$) chromosome to the *sat* line, the mutator element was remobilized, resulting in approximately 50 lines with white eyes. sat^{15} and sat^{r2} are representative of these lines: sat^{15} is lethal when expressed homozygously and induces mutant phenotypes when expressed heterozygously with *sat*, whereas sat^{r2} does not induce mutant phenotypes. We consider sat^{r2} to be a revertant because precise excision of the PlwB insertion from the genome was confirmed by Southern analysis.

To quantify the intensity of courtship activity displayed by a given male, we used the sex appeal parameter (12) index (SAPI), which is defined as the fraction of time the male exhibits unilateral wing vibration in the total observation period. For instance, singing by a male for 3–4 min in a 10-min observation period gives a SAPI of 30–40. Some researchers have used the courtship index in studies in which the duration of the entire courtship sequence was measured as the courtship time. Since it is sometimes difficult to judge whether the males are sexually motivated when they orient their bodies toward females and follow the females, the SAPI, which provides an unequivocal estimation of the courtship activities of the males, was used. It is known that males reject courtship by other males by rapidly flicking both of their wings. Such bilateral wing displays are easily distinguishable from courtship and are excluded from the SAPI measurement.

The dorsal muscles including the MOL were stained with fluorescein isothiocyanate-labeled phalloidin.

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Abbreviations: MOL, muscle of Lawrence; SAPI, sex appeal parameter index; RACE, rapid amplification of cDNA ends.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D84437 and D84438).

*Present address: Central Institute for Experimental Animals, 1430 Nogawa, Miyamae-ku, Kawasaki, Kanagawa 216, Japan.
†To whom reprint requests should be addressed.

Expression Analysis. The brain was dissected out from *sat* flies and was double stained with an anti-β-galactosidase antibody and a nuclear stain, DiI. The stained brain was observed with a laser confocal microscope, and images of it were subjected to three-dimensional reconstruction.

Molecular Analysis. The plasmid rescue method was used to recover the *sat* genomic DNA flanking the P-element insertion point. Methods for extraction and analysis of RNA and genomic DNA were as described elsewhere (13). Using *Drosophila* head and pupal cDNA libraries, 1×10^6 phages were screened, and several *fru* cDNAs were isolated. The nucleotide sequences of the cDNAs were determined using a 377 DNA sequencer (Applied Biosystems Prism). *In situ* hybridization to the brain and to polytene chromosomes was performed as described elsewhere (13).

RESULTS

Behavioral Phenotypes of the *sat* Mutant. The *sat* mutant male and female pairs did not copulate in the 1-h observation period. Likewise, the *sat* males paired with Canton-S wild-type females did not mate (Fig. 1*A*), whereas the *sat* females were highly receptive to courting by Canton-S males. Thus the nonmating phenotype is due to a defect in the male. The SAPI estimated for the *sat* males was zero (Fig. 1*B*), and that estimated for the Canton-S males was about 38, regardless of the genotype of the target mature females (Fig. 1*B*). This suggests that the *sat* males are not motivated to court females or that their courting behavior is somehow blocked. A fragment of the PlwB element was used as a probe for *in situ* hybridization to *sat* chromosomes, whereby the PlwB element insertion site was identified at 91B, at which *fru* was mapped (6). The fru^1 males are known to court other males as well as females (5, 6), although they do not copulate (Fig. 1*A*). To investigate whether *sat* is allelic to *fru*, mating behavior of sat/fru^1 transheterozygotes was examined. In addition, the deletion chromosomes that bear break points in this region were placed in *trans* to the *sat* insertion to test for their ability to block copulation. In spite of the fact that both *sat* and fru^1 mutations prevent mutant males from copulating (Fig. 1*A*), the transheterozygous males mate with females, although less often than the wild-type flies do (Fig. 1*A*). Besides in fru^1 and *sat* homozygotes, complete block of copulation was attained only when the *sat* insertion was placed in *trans* to $Df(3R)Cha^{M7}$, the largest deletion amongst those used in this experiment (Fig. 1*A*). Although the sat/fru^1 males mate with females and are fertile, they display strong male-male interactions, forming so-called courtship chains (Fig. 1*C*), in which a courting male is courted by other males resulting in a long line of suitors (6). Both *sat* and fru^1 homozygous males engage in courtship chain formation. The fact that the *sat* mutation does not affect the fru^1 bisexual courtship behavior suggests the existence of an allelic relationship between *sat* and fru^1.

MOL in *sat*. Further support for the hypothesis that *sat* is allelic to *fru* comes from the observation that *sat* homozygous males lack a pair of large sex-specific muscles, the MOL in their abdomen (Fig. 2*A* versus *B*). The MOL exists in the sat^{r2} revertant (see *Materials and Methods*) from whose genome the *sat* insertion has been excised (Fig. 2*D*) and is not formed in flies having two overlapping deletions covering the entire *fru* locus (9). The recently isolated fru^3 mutant (7, 14) also lacks the MOL (Fig. 2*C*). We conclude that *sat* is a new allele of the *fru* locus and refer to *sat* as fru^{sat} hereafter.

The Molecular Nature of *fru* and Its Products. Starting from the fru^{sat} insertion point, we walked over 90 kb of the genomic DNA and mapped several rearrangements linked to the *fru* locus (Fig. 3*A*). The distal break point of *Df(3R)P14* (90C2-D1;91B1-2) was mapped 7 kb proximal to the fru^{sat} insertion point, without removal of DNA at the insertion point itself. fru^{sat15} (see *Materials and Methods*) is a lethal allele resulting from imprecise excision of the fru^{sat} insertion and has a deletion of at least 17 kb proximal to the insertion

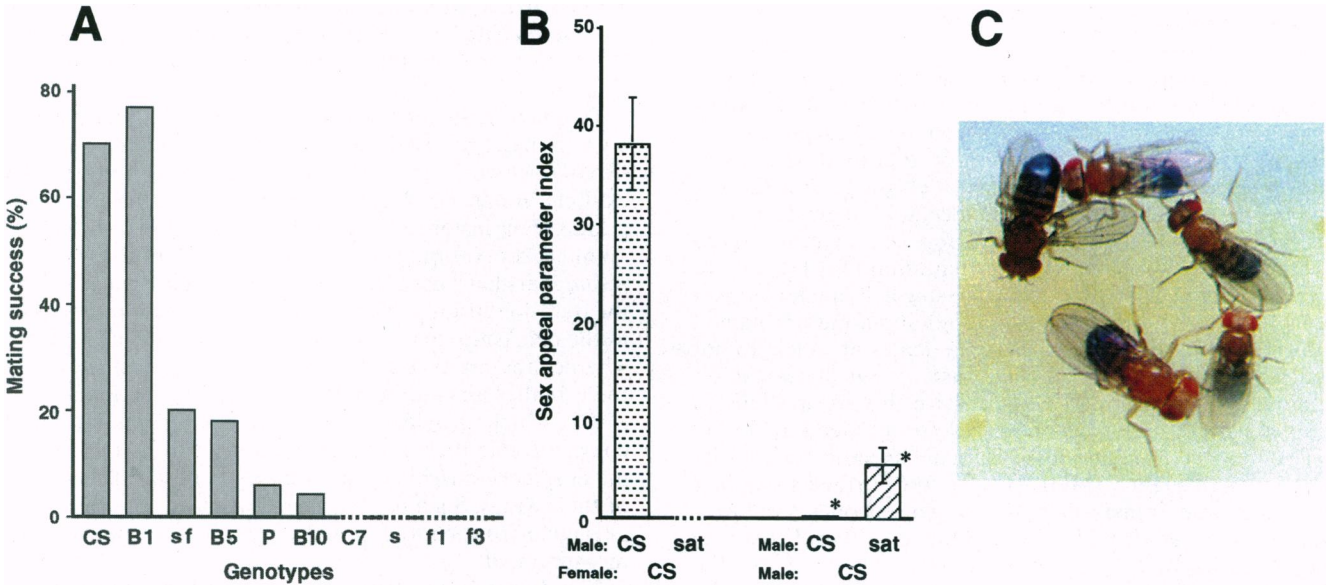


FIG. 1. Behavioral phenotypes of the *sat* and related mutants. (*A*) The percentage of single mutant male and Canton-S female pairs that copulated in the 1-h observation period is shown as mating success. The genotypes of males examined are Canton-S (CS), $sat/Df(3R)BX1$ (B1), sat/fru^1 (sf), $sat/Df(3R)BX5$ (B5), $sat/Df(3R)P14$ (P), $sat/Df(3R)BX10$ (B10), $sat/Df(3R)Cha^{M7}$ (C7), *sat* (s), fru^1 (f1), and fru^3 (f3). Twenty pairs were examined for each genotype. The deleted interval for each deficiency is as follows (7): $Df(3R)BX1$ (90F8-11;91B1-2), $Df(3R)BX5$ (91B1-2;91D1-2), $Df(3R)P14$ (90C2-D1;91B1-2), $Df(3R)BX10$ (90C7-8;91B1-2), and $Df(3R)Cha^{M7}$ (90F;91F). (*B*) The SAPIs (see *Materials and Methods*) estimated for *sat* homozygous males and Canton-S males when paired with Canton-S females (left two bars) or with Canton-S males (right two bars) are compared. The average and the standard error of the mean of 20 independent measurements with different individuals are shown for each case. All flies used were 3 days old. The genetic background of the *sat* mutant line was standardized to Canton-S by five-generation outcrosses to Canton-S. The asterisks indicate that the difference is statistically significant ($P < 0.001$, Student's *t* test). (*C*) The courtship chain formed by sat/fru^1 males in the absence of females.

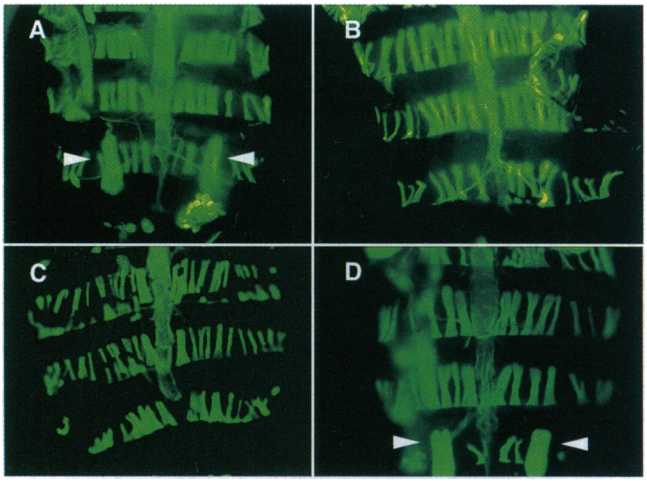


FIG. 2. The MOL in male Canton-S (*A*), fru^{sat} (*B*), fru^{3} (*C*), and fru^{satr2} (*D*) flies.

point. In $Df(3R)Cha^{M7}$ (90F;91F) flies, the entire 90-kb region of the cloned genome DNA is deleted. The fru^{3}

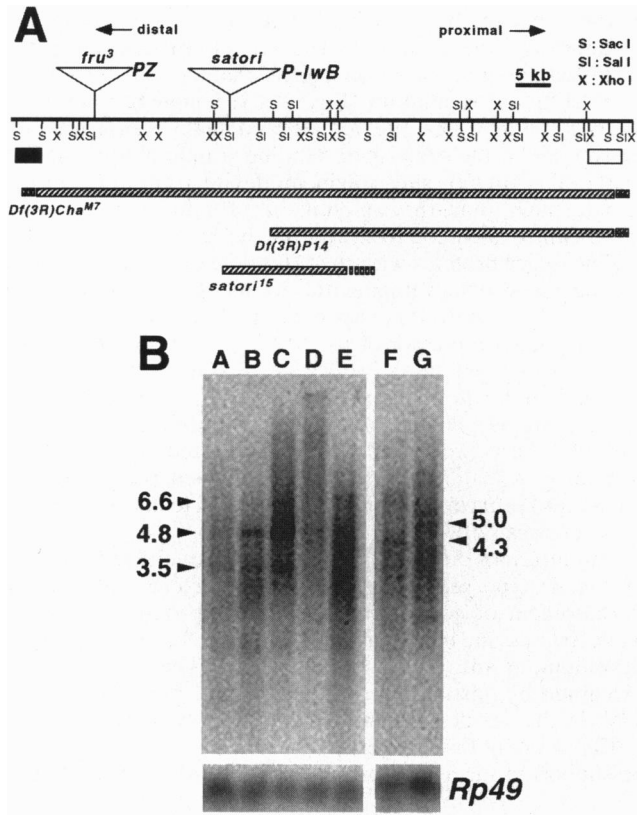


FIG. 3 Molecular analysis of the *fru* locus. (*A*) The P-element insertion sites in fru^{sat} and fru^{3} are indicated with triangles. Below the restriction map, deleted regions in the fru^{sat15}, *Df(3R)P14*, and $Df(3R)Cha^{M7}$ chromosomes are indicated by the hatched bars. The genomic DNA fragments were used as probes to screen cDNA libraries. The probe shown with an open box yielded a cDNA clone for a *fru* transcript. The filled box indicates a genomic fragment that hybridizes to the 5′-RACE product containing the Tra binding sites. (*B*) Northern blot of wild-type mRNA. Poly(A)$^+$ RNA was isolated from embryos (lane A), second instar larvae (lane B), early third instar larvae (lane C), late third instar larvae (lane D), pupae (lane E), and male (lane F) and female (lane G) adults. The ribosomal protein 49 (*Rp49*) cDNA was used as a control probe. The bands indicated with the arrow heads were reproducibly identified in different blotting.

insertion is located 21 kb distal to the fru^{sat} insertion site. The $fru^{1}/Df(3R)P14$ (6), $fru^{1}/Df(3R)Cha^{M7}$ (6), fru^{3} (7), and fru^{1}/fru^{sat15} males (D.Y., unpublished observation) all exhibit homosexual courtship activity. Taken together, the above findings suggest that the cloned genomic DNA contains at least part of *fru*.

To identify the *fru* transcription unit, larval, pupal, and adult poly(A)$^+$ RNA was probed with genomic DNA fragments derived from the cloned region on Northern blots. A genomic probe derived from the region 50 kb proximal to the fru^{sat} insertion hybridizes with several transcripts, including those which are sexually dimorphic in terms of their size. The 4.3-kb mRNA is unique to males, whereas the 5-kb mRNA is found only in females (Fig. 3*B*). Screening of *Drosophila* adult head and pupal cDNA libraries yielded cDNAs with a 3-kb insert, which contained a polyadenylylation signal at its 3′-end. To cover the full length of the transcripts, we used 5′-rapid amplification of cDNA ends (5′-RACE) with mRNA extracted from either males or females as a template. We obtained 4 types of 5′-RACE products from male mRNA and 3 from female mRNA. One of them was common to both sexes. One female-type 5′-RACE product hybridized with a DNA fragment at the distal end of our chromosomal walk (Fig. 3). Furthermore, through partial sequencing of the genomic DNA flanking the fru^{3} insertion site, we identified an approximately 100-bp stretch identical to one in a 5′-RACE product. We consider this to be the *fru* transcription unit because (*i*) its intron contains the fru^{sat} and fru^{3} insertions as well as deficiencies that are responsible for the *fru* phenotypes, (*ii*) one of its exons is located very close to the mutagenic P-element insertion, and (*iii*) it shows sex-dependent variations.

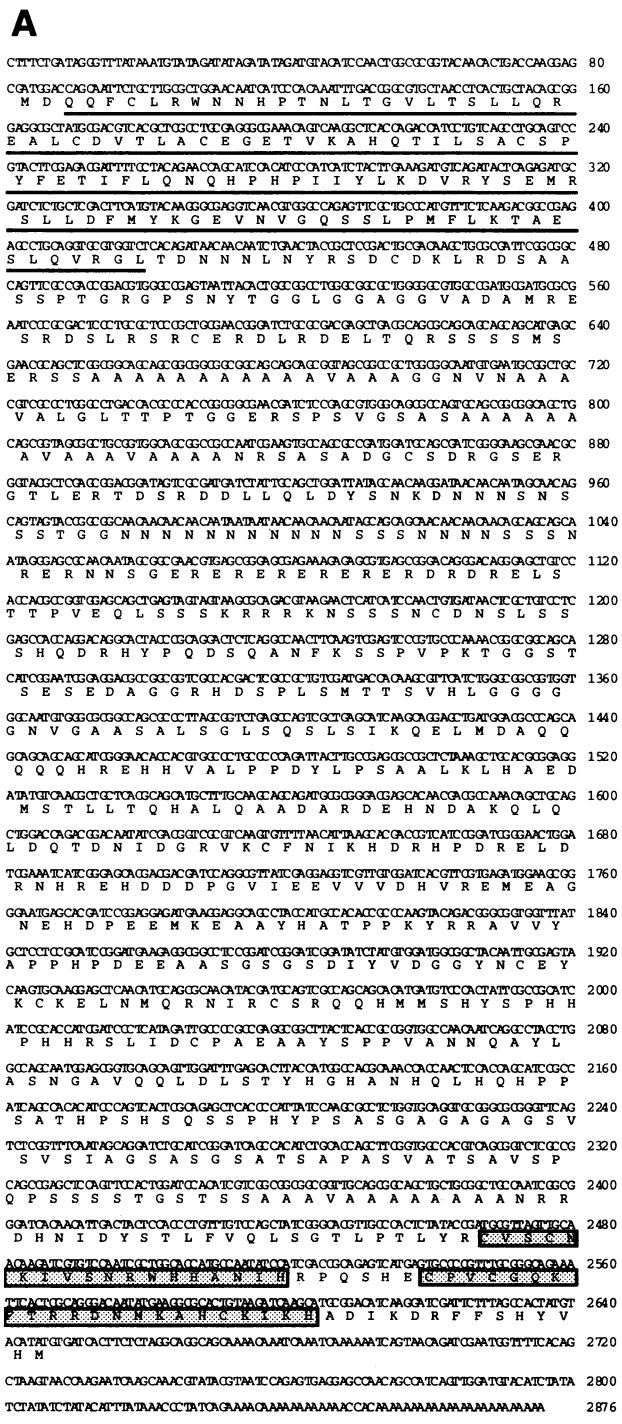
Sequence analysis revealed that the cDNAs contain a long open reading frame that potentially encodes a protein of 855 amino acid residues (Fig. 4*A*) with a BTB domain (Fig. 5*A*) and two zinc finger motifs (Fig. 4*A*). The sequence of a 5′-RACE product from female mRNA contains three 13-nucleotide repeats (Fig. 5*B*) similar to those found in the doublesex (*dsx*) transcript, where each repeat functions as a binding site for Tra to induce sex-specific splicing of the *dsx* primary transcript (15). The structure of the *fru* product suggests that it is a transcription factor acting downstream of Tra. Males and females have other types of transcript that lack the putative Tra-binding site at their 5′ terminus.

***fru* Expression Patterns.** The pattern of *fru* mRNA expression was examined by digoxigenin *in situ* hybridization of whole-mount preparations of brain–ganglion complexes isolated from third instar larvae. A cDNA fragment whose sequence is common to both sexes was used as a probe. The transcript was localized in the mushroom body and the optic lobe (Fig. 6*A*) in the brain. Some cells in the ganglion were also stained (Fig. 6*A*).

The *lacZ* reporter in the P-element vector was used to investigate *fru* expression patterns in the adult fru^{sat} brain because of technical difficulties in staining the adult brain by means of *in situ* hybridization. No difference was detected in the staining patterns between homozygotes and heterozygotes. *lacZ* expression was detected in the cells at the frontal surface of the dorso-central brain and in the antennal lobe cells (Fig. 6*B*). We were unable to detect any sex differences in the spatial expression of the *fru* gene in this experiment.

DISCUSSION

We have shown that the homosexual mutant *sat* has a deficit in *fru* gene function. Different alleles of *fru* display a spectrum of mating phenotypes in males, i.e., homosexual, sterile bisexual, and fertile bisexual. The fru^{sat} allele is unique in that the males carrying it do not court mature females (Fig.



1*B*) unlike other *fru* mutant males that court both mature males and females (6, 7). Sterility seems to result totally from block of copulation, because heteroallelic mutant males, fru^1/fru^{sat}, copulate with females (Fig. 1*A*) and produce progenies, although males homozygous for fru^1 or fru^{sat} are completely sterile. In addition, the shape, number, and mobility of sperm in fru^{sat} homozygous males are normal (H.I., unpublished data), suggesting that reproductive physiology is intact in the *fru* mutants. The variation in the mating phenotypes might reflect differences in the amount of the functional gene product remaining in the mutants and/or different effects of the alleles on expression pattern of the *fru* gene.

The known mutations at the *fru* locus induce a range of defects in MOL development (7–9). fru^{sat} is a strong allele in that all males carrying it lack the MOL (Fig. 2). MOL formation is known to depend on activity of the sex-determination cascade mediated by Sexlethal (*Sxl*), *tra*, and *tra-2*, but is independent of *dsx* (16). The chromosomally female (XX) flies that are mutant for either *Sxl*, *tra*, or *tra-2* have a MOL-like muscle, whereas *dsx* females do not, suggesting that *fru* and *dsx* each contribute to a different branch of the bipartite pathways downstream of *tra* (17, 18). Each pathway might regulate mutually exclusive sets of target genes necessary for development of sexually dimorphic characteristics. The structure of the *fru* transcript is fully consistent with this idea in that it has three putative Tra binding sites and is predicted to encode proteins with the BTB domain and zinc finger motifs characteristic of several transcriptional regulators (Fig. 5). Although the DNA sequences from which the female and male transcripts are derived share the same open reading frame, binding of Tra to the Tra binding sites might modulate translation in the female such that the amount of protein synthesized is significantly different from that in the male.

Zinc finger proteins with the BTB domain are required for a wide range of developmental events. They can be either transcriptional activators or repressors. For example, Ttk is a transcriptional repressor of pair-rule genes, fushi tarazu (*ftz*) and even-skipped (*eve*), while the GAGA factor activates Krüppel and *Ubx* transcription through an antirepression mechanism (19). Results of experiments with transfected cells and the yeast two-hybrid system suggested that the BTB domain in a mammalian zinc-finger protein (LAZ3/BCL6) homomerizes *in vivo* and targets the protein to discrete nuclear substructures (20).

dsx mutations do not impair expression of either male-type or female-type sexual behavior yet interfere with normal development of genital structure and reproductive organs, while *Sxl*, *tra*, and *tra-2* mutations all affect both sexual organ development and sexual behavior (21). This can be easily explained by postulating the existence of bifurcating pathways in the sex-determination cascade downstream of *tra*.

The idea that Fru is a neuronal counterpart of Dsx seems to be supported further by its effect on the MOL; the MOL is

FIG. 4. Structure of *fru* cDNAs. (*A*) The nucleotide sequence of a cDNA and the amino acid sequence predicted from it. (*B*) The 5′-end of the cDNA obtained by 5′-RACE using mRNA extracted from adult female flies as a template. The 5′-RACE product with putative Tra binding sites was derived only from female mRNA, suggesting that it represents the female-specific forms of the *fru* products. G at nucleotide position 685 of the 5′-RACE product (*B*) was replaced with A in the cDNA clone (*A*, nucleotide 61). The sequences upstream of nucleotide 592 (numbering of the RACE product nucleotide sequence) were different between the cDNA and RACE products, reflecting different exon uses. A fragment composed of 27 nucleotides (770–796 in *B*) was used as the primer for 5′-RACE. The putative Tra binding sites are boxed. The BTB domain is underlined, and the zinc finger motifs are shown with shaded boxes.

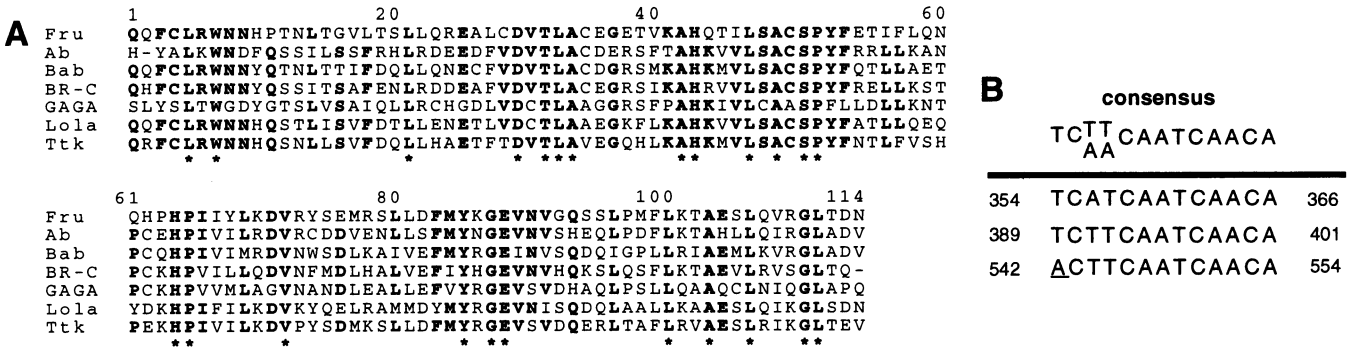


FIG. 5. Alignment of conserved amino acid sequences in the BTB domain (*A*) and nucleotide sequences at the putative Tra binding sites (*B*). Fru is compared with Abrupt (Ab), Bric à brac (Bab), BR-C (Broad-complex), GAGA transcription factor (GAGA), Longitudinals lacking (Lola), and Tramtrack (Ttk) (see refs. 10 and 25). The amino acid residues that are identical for 5 of 7 proteins are shown in boldface, and the residues conserved in all proteins are indicated with asterisks. The nucleotide numbers in (*B*) correspond to those in Fig. 4*B*.

formed even if the contributing myoblasts are female (22), provided the innervating nerve is male (23, 24). Thus, the MOL phenotype associated with the *fru* alleles is presumably ascribable to a deficit in the motoneurons.

The altered sexual orientation in the *fru* mutants likely results from incorrect sex determination of neural cells (17, 18). It is interesting that Fru expression as detected by staining of enhancer-trap reporters was observed in a subset of cells in the antennal lobe of the adult brain (Fig. 6). It was recently shown that male to female transformation of cells in some glomeruli of the antennal lobe by means of GAL4-driven targeted expression of tra^+ can alter sexual orientation in males of *D. melanogaster* from heterosexual to bisexual (1). These findings prompt us to speculate that the *fru*-expressing cells in the antennal lobe are involved in the determination of sexual orientation.

Since the antennal lobe is the primary olfactory center that receives afferents from receptors on the antennae and maxillary palps (25), it is plausible that neural processing of odor cues for sexual recognition in this center is impaired in *fru* mutants. Incorrect interpretations of odor cues may lead to failure in initiating sexual behavior toward females and trigger inadequate courtship toward males.

Sex is determined by diverse mechanisms that vary among species. Despite fundamental differences in sex-determination mechanisms, the physiological outcomes of dysfunctioning of these mechanisms can be quite similar in different animal species. The present findings raise the intriguing possibility that mutations in a single sex-determination gene result in sexual transformation of a subset of brain cells, thereby altering sexual orientation in the male.

Note Added in Proof. Since submission of the manuscript, we have isolated cDNA clones that potentially encode four additional variants of Fru.

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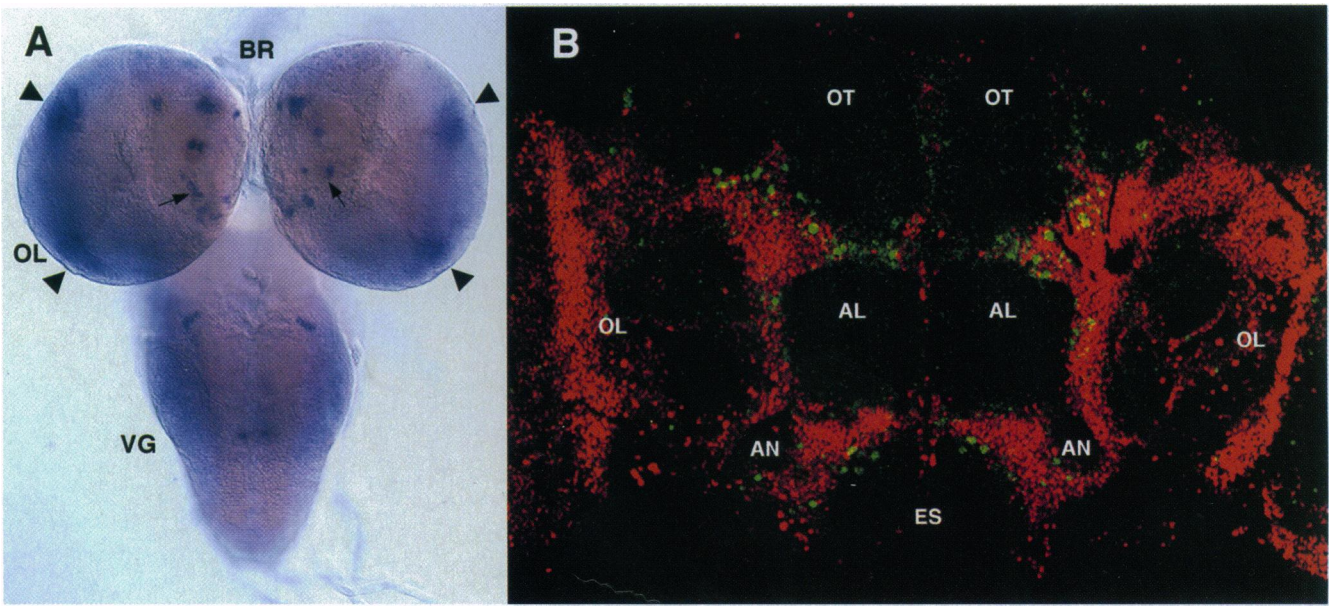

FIG. 6. The *fru* expression patterns. (*A*) A male larval brain-ganglion complex subjected to whole mount *in situ* hybridization. The digoxigenin-labeled *fru* cDNA probe reveals *fru* mRNA expression in the mushroom body (arrows) and the optic lobe (arrowheads). (*B*) A confocal image of a frontal optic section of the adult male brain (approximately 9 μm from the brain surface). DiI nuclear staining is indicated by red, and *lacZ* expression is indicated by green. The antennal lobe (AL), antennal nerve (AN), brain (BR), esophagus (ES), optic lobe (OL), optic tubercle (OT), and ventral ganglion (VG) are indicated. *lacZ* expression was detected by immunocytochemistry with an anti-β-galactosidase antibody.

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