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Genes expressed in the *Drosophila* head reveal a role for fat cells in sex-specific physiology

Shinsuke Fujii and Hubert Amrein[1]

Department of Molecular Genetics and Microbiology,
Duke University Medical Center,
252 CARL Bldg/Research Drive, Durham, NC 27710, USA

[1]Corresponding author
e-mail: hoa1@duke.edu

The downstream effectors of the *Drosophila* sex determination cascade are mostly unknown and thought to mediate all aspects of sexual differentiation, physiology and behavior. Here, we employed serial analysis of gene expression (SAGE) to identify male and female effectors expressed in the head, and report 46 sex-biased genes (>4-fold/$P < 0.01$). We characterized four novel, male- or female-specific genes and found that all are expressed mainly in the fat cells in the head. *Tsx* (*turn on sex-specificity*), *sxe1* and *sxe2* (*sex-specific enzyme 1/2*) are expressed in males, but not females, and are dependent on the known sex determination pathway, specifically *transformer* (*tra*) and its downstream target *doublesex* (*dsx*). Female-specific expression of the fourth gene, *fit* (*female-specific independent of transformer*), is not controlled by *tra* and *dsx*, suggesting an alternative pathway for the regulation of some effector genes. Our results indicate that fat cells in the head express sex-specific effectors, thereby generating distinct physiological conditions in the male and female head. We suggest that these differences have consequences on the male and female brain by modulating sex-specific neuronal processes.

Keywords: *Drosophila*/fat cells/mating behavior/SAGE/sex-specific genes

Introduction

In *Drosophila*, sex determination is mediated by the ratio of X chromosomes to sets of autosomes (X:A ratio). A ratio of 1 (2X:2A) initiates a regulatory cascade involving the splicing regulators *Sex-lethal* (*Sxl*), *transformer* (*tra*) and *transformer2* (*tra2*), and ultimately leads to the generation of sex-specific transcription factors encoded by *doublesex* (*dsx*) and *fruitless* (*fru*; reviewed by McKeown, 1994; Cline and Meyer, 1996). *Sxl* is transcriptionally active in both sexes, but only the female embryos produce functional SXL protein by a mechanism of alternative splicing which is maintained by an autoregulatory feedback mechanism (Bell *et al*., 1991). SXL also regulates female-specific splicing of *tra* pre-mRNA to allow production of functional TRA protein (Sosnowski *et al*., 1989; Inoue *et al*., 1990). TRA and the constitutively expressed TRA2 (Amrein *et al*., 1988; Goralski *et al*., 1989) positively regulate *dsx* pre-mRNA splicing to generate a female-specific transcript encoding DSX^f protein (Burtis and Baker, 1989). In males, which have an X:A ratio of 0.5 (1X:2A), the absence of SXL, and consequently of TRA, results in constitutive splicing of *dsx* pre-mRNA to produce a transcript that encodes a male-specific protein, DSX^m. The absence of TRA in males also results in the alternative splicing of *fru* pre-mRNA, leading to expression of a male-specific FRU^m protein, which is essential for female-directed male courtship behavior (Ryner *et al*., 1996). The function of TRA and TRA2 for female differentiation is dramatically revealed in XX flies homozygous mutant for either gene; these flies are sexually transformed and develop as so-called ψ (pseudo)-males, which are indistinguishable from normal XY males in morphology and behavior, but are sterile (Baker and Ridge, 1980; Belote and Baker, 1987). Three other regulatory genes, *intersex* (*ix*), *hermaphrodite* (*her*) and *dissatisfaction* (*dsf*), are also involved in the regulation of sex determination. However, they are likely to mediate their male and female functions through other sex-specific factors, since at least HER and DSF appear to be expressed equally in both sexes (Chase and Baker, 1995; Finley *et al*., 1998; Li and Baker, 1998).

Whereas the regulatory splicing pathway of *Sxl–tra–dsx/fru* is well understood, much less is known about the downstream targets (effectors). The only sex-specific effectors identified to date are the female-specifically expressed yolk protein genes *yp1*, *yp2* and *yp3*, at least two of which (*yp1* and *yp2*) are directly regulated by the DSX proteins (Burtis *et al*., 1991; Coschigano and Wensink, 1993; An and Wensink, 1995a,b). DSX^f and DSX^m share identical DNA-binding domains, but differ in their C-termini, which function as protein–protein interaction domains (Coschigano and Wensink, 1993; Erdman and Burtis, 1993). This observation, together with *in vivo* analysis of *yp1* promoter–lacZ reporter constructs (Abrahamsen *et al*., 1993), has led to a model in which DSX^f acts as a transcriptional activator and DSX^m as a repressor of the *yp1* and *yp2* genes. No other downstream target genes of DSX or FRU are known.

To understand the molecular, sex-specific processes that operate during development and control adult behavior, genome-wide expression analysis will be necessary. Here we report a first step towards this goal. To identify effector genes with sex-specific or sex-biased expression in the adult, we carried out serial analysis of gene expression (SAGE) and generated a transcript inventory of the *Drosophila* male and female head. Analysis of >7000 unique tags (i.e. transcripts) revealed the presence of 46 sex-biased candidate genes. Northern blot analysis of eight novel candidates confirmed sex specificity/bias for all of them. Interestingly, four of the genes were expressed in either males or females only, most notably in the fat cells of the head. Three of these genes are virtually male specific and are dependent on the sex determination

cascade, particularly on *dsx*. Surprisingly, expression of the fourth gene, which is highly female biased, is dependent on *Sxl*, but independent of *tra*, *tra2* and *dsx*, suggesting the existence of an alternative pathway important for sex-specific gene expression. We propose that the fat cells in the head, and perhaps other non-neuronal cells, play an important role in establishing sex-specific differences in physiology, which might modulate brain function and ultimately influence male and female behaviors, including courtship and mating.

Results

We sought to identify genes that are expressed at significantly different levels in adult males and females. We reasoned that SAGE (Velculescu *et al*., 1995) could be used efficiently to investigate differences in gene expression in the male and female head and, thus, should allow us to identify sex-specific genes that might be involved in behavior. We decided to use RNA from heads, as opposed to whole flies or dissected brains, for two main reasons. First, it eliminates a large number of genes expressed in the testes and ovaries. Secondly, even though the brain plays the central role in behavior, it may not necessarily be the major source of male- and female-specific genes, but rather be modulated by 'instructive signals' such as peptides and/or hormones synthesized in other tissues. Therefore, we generated SAGE libraries from RNA of wild-type male and female heads and compiled a data set of >110 000 individual transcripts (67 000 female tags and 49 000 male tags) representing ~7000 genes (for details, see Materials and methods). The vast majority of tags are present at similar levels in both sexes (Figure 1A).

A small set of sex-biased transcripts identified by SAGE

We focused our studies on genes that were expressed at least 4-fold more abundantly in one sex versus the other. Applying moderate ($P < 0.01$) or stringent ($P < 0.001$) probability criteria, we identified 46 or 13 genes, respectively (Figure 1A; Table I; see Materials and methods), including all five previously known sex-specific genes, the three *yolk protein* (*yp*) and both *roX* genes. *roX1* and *roX2* were found exclusively in males and were represented by 13 and 12 tags, respectively. (Note that throughout this paper, the terms 'sex-specific', 'male-specific' and 'female-specific' refer to expression differences of at least 10-fold between the sexes as analyzed by northern blot analysis and phosphoimaging.) Tags for the *yp* genes were found several hundred times in the female library, but only once or never in the male library (Table I). Sex-biased expression of the eight novel candidates was confirmed by northern blot analysis on male and female head RNA (Figure 1B). We focused our investigations on the four novel, sex-specific genes *CG7592*, *CG17820, cyp4d21* and *CG4979*. The entire data set from our SAGE can be accessed at http://rush.genetics.duke.edu/amrein/sage/

The tags representing the male-specific genes encode members of three distinct protein families (Table I): an odorant-/pheromone-binding protein (opbp; CG7592), a cytochrome P450 enzyme (CYPs; *cyp4d21*) and a phospholipase (CG4979). The female-specific tag identified a gene of unknown biochemical function (CG17820). We shall refer to these genes as *turn-on sex-specificity* (*tsx*), *sex-specific enzyme 1* and *2* (*sxe1* and *sxe2*) and *female-specific independent of transformer* (*fit*), respectively.

Tissue and developmental expression of the sex-specific genes

We investigated the tissue-specific expression of *tsx*, *sxe1*, *sxe2* and *fit* using northern blot hybridization with RNA isolated from the head, thorax and abdomen of male and female adult flies (Figure 2A). Northern blot analysis confirmed our results from the SAGE screen; all four genes were expressed in the head in a sex-specific manner. In addition, *tsx* was expressed in all major body segments of the adult, whereas *sxe1* and *fit* are expressed at much lower levels in the thorax than the head and not at all in the abdomen. Finally, *sxe2* is expressed exclusively in the male head. Initially, *in situ* hybridization experiments for all four genes did not show defined and localized signals within the head, whereas control experiments with the male-specific *roX2* gene (Amrein and Axel, 1997), the neuron-specific *synaptobrevin* gene (*n-syb*; Sudhof *et al*., 1989) and the fat body-specific *yp3* gene (Garabedian *et al*., 1987) revealed the expected sex and tissue specificity (Figure 3, right panel; data not shown). This observation suggested that the expression levels of the novel genes are too low for detection by this technique. However, during the course of this study, we identified two strains (w^{1118} and B^s*;CyO/cn tra2 bw*) in which *tsx* and *fit* are expressed at significantly higher levels than in wild-type *Ore-R* flies (Figure 3C). We therefore performed *in situ* hybridization experiments on tissue sections of fly heads from these strains for both *tsx* and *fit*. Hybridization signals of *tsx* transcripts were localized to the (deep) fat cells in the ventral and dorsal regions of the head but not the brain, the eyes, the antenna, the maxillary palps or the labellum of *CyO* males or *tra2* ψ-males (Figure 3A, left panel, and B). As expected, no hybridization signals were observed in any parts of female heads (Figure 3A, bottom of left panel). For *fit*, we used heads from males and females of the w^{1118} strain (Figure 3A, middle panel). Hybridization signals were only found in females, but not males, and were also restricted to deep fat cells in both the dorsal and ventral part of the head. Again, we noticed no *fit*-positive cells in the brain, the visual or the chemosensory systems. Thus, these data showed that *tsx* and *fit* are expressed mainly in the fat cells of the male or female head, respectively.

To examine tissue specificity further, we performed northern blot analysis of RNA from dissected heads (Figure 2B). The 'purity' of the dissected tissue was examined by northern hybridization experiments with probes for tissue-specific genes, which demonstrated that the desired tissue was either successfully enriched for or removed. For example, brain-specific *n-syb* transcripts (Sudhof *et al*., 1989) were highly enriched in dissected brain (B) when compared with carcass (C), whereas fat body-specific *yp3* expression (Garabedian *et al*., 1987) was found abundantly in the carcasses and heads lacking chemosensory organs (H^-), but not the brain. Similarly, *pbprp2* expression, which is restricted to the chemosensory system (Pikielny *et al*., 1994), was almost entirely lost in the H^- fraction (Figure 2B, lower panels). When these

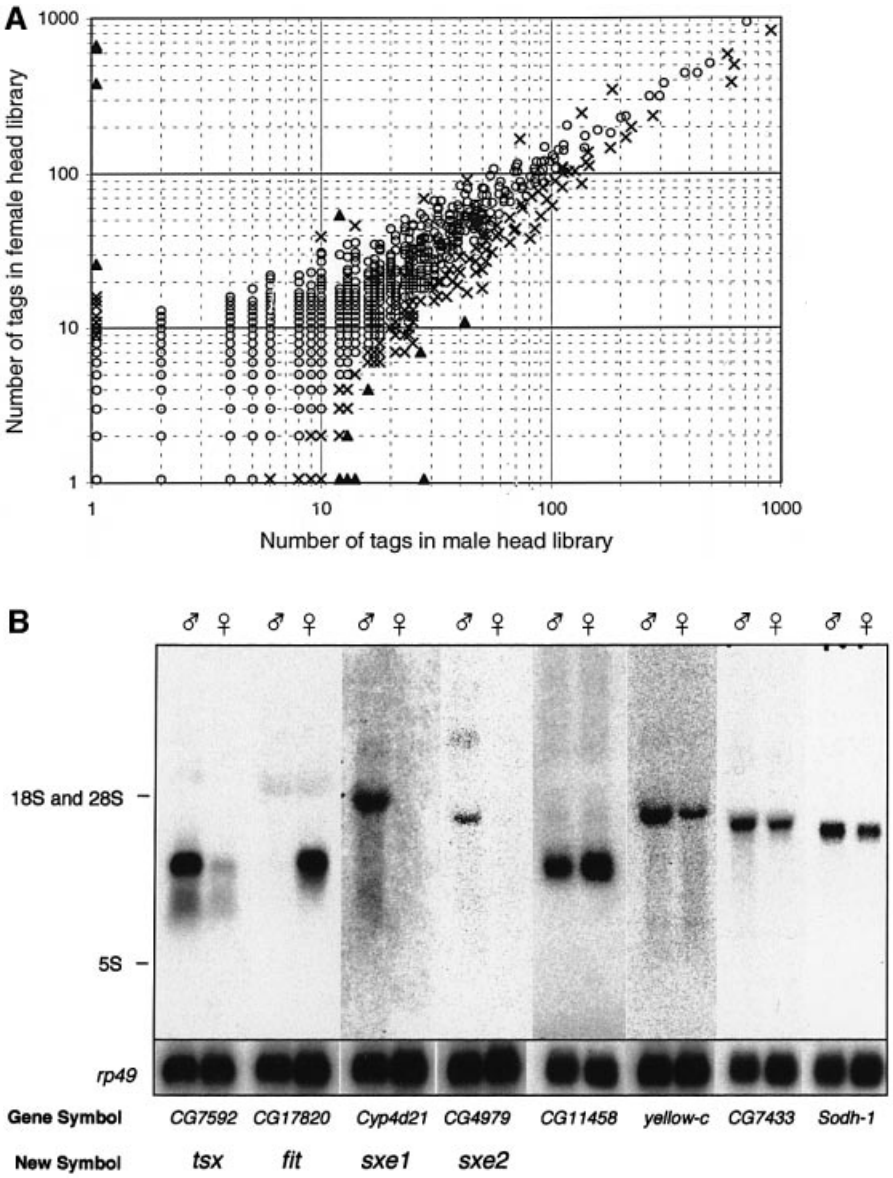


Fig. 1. Identification of sex-biased SAGE tags and expression of candidate genes. (**A**) Distribution of SAGE tags from male and female head libraries. The number of times each unique SAGE tag was observed was plotted on a logarithmic scale. Tags not found in a library were converted to a value of 1. Male tag numbers are normalized to female tag numbers (coefficient is 1.4). Triangles indicate tags with $P < 0.001$; crosses indicate tags with $0.001 \leqslant P < 0.01$; circles indicate tags with $P \geqslant 0.01$. (**B**) Northern blot analysis of eight novel, sex-biased genes in male and female heads. Total RNA was isolated from dissected male and female heads. Blots were hybridized with probes to all eight novel genes, represented by SAGE tags that are at least four times more abundant in one library versus the other (≥4-fold; $P < 0.001$). 18S/28S and 5S RNA markers are indicated on the left and were derived from ethidium bromide-stained gels. All blots contained 5 μg of male and female RNA per lane, respectively, and were re-hybridized with a probe against the *rp49* gene to provide an RNA loading control.

northern blots were hybridized with probes for *tsx*, *sxe1*, *sxe2* and *fit*, we found that none was expressed in the brain (Figure 2B, top panels, lane B). All genes were expressed in the C and H^- preparations, suggesting that the head carcass, but not the chemosensory system, is the main source of transcripts for all four genes (Figure 2B, top panels, lanes C and H^-). These findings are consistent with the *in situ* hybridization experiments that detected *tsx* and *fit* transcripts in the deep fat cells in the head (Figure 3A).

To investigate whether *tsx*, *sxe1*, *sxe2* and *fit* are expressed exclusively in adults, we hybridized probes for each of them to RNA from various embryonic, larval and pupal stages. The only gene expressed prior to the adult stage was *tsx*, which was first observed in the third instar larvae, but then gradually decreases during pupal stages (data not shown). Interestingly, expression in larvae is non-sex specific and higher than in adult males.

The male-specifically expressed genes are dependent on tra, tra2 and dsx function

To explore the regulation of the four sex-specific genes, we carried out a series of northern blot analyses using flies with mutations in various sex determination genes. For example, XX animals homozygous mutant for *tra* or *tra2* are sexually transformed into ψ-males, which are indistinguishable from wild-type, *tra* or *tra2* XY males in their sexual morphology and behavior. Thus, male-specific genes that are downstream of *Sxl* and *tra/tra2* in the sex

Table I. List of sex-biased tags in the adult fly head

Tag sequence	Male	Female	*P* chance	Gene symbol	Function/note	CG number	Location
GCCACGCCCC	0	667	< 0.0001	***Yp3***	Yolk protein	11129	12B8
CGAGCGAACC	0	644	< 0.0001	***Yp2***	Yolk protein	2979	9B1
GGCATCGATA	1	381	< 0.0001	***Yp1***	Yolk protein	2985	9B1
GATCCAGCCA	28	1	< 0.0001	*CG7592 (tsx)*[a]	Ligand binding or carrier	7592	99B8
TCATTCATTC	0	26	< 0.0001	*CG17820 (fit)*[a]	Unknown	17820	93F8–9
CCAGGAGCAA	14	0	< 0.0001	*Cyp4d21 (sxe1)*[a]	Cytochrome P450	6730	28B4
TTTTGACAGA	13	0	< 0.0001	***roX1***	Non-coding RNA, dosage compensation	–	3F
GTTGACGCGC	12	0	< 0.0001	***roX2***	Non-coding RNA, dosage compensation	–	10B17
GGCGGTGAAC	13	2	0.0005	*CG4979 (sxe2)*[a]	Phosphatidylserine-specific phospholipase A1	4979	89B6
GAGGCGGCGG	12	54	< 0.0001	*CG11458*[a]	Unknown	11458	77F4
CTGTGTTTAT	16	4	0.0008	*yellow-c*[a]	*yellow* family	4182	35B8
CCAACATCGT	27	7	< 0.0001	*CG7433*[a]	4-aminobutyrate aminotransferase	7433	76D8–E1
ATCCACGTCC	42	11	< 0.0001	*Sodh-1*[a]	L-iditol 2-dehydrogenase	1982	84B2
CACTTCGTAG	8	0	0.001	*CG15219*[b]	Testis-specific transcript	15219	40B1
GCCTATTCGT	8	0	0.001	*AI946554*[b]	Testis-specific transcript	–	60E12
TAATAACAAC	10	1	0.001	*CG10135*	Calcium binding, calcium sensing	10135	87D1
TGCGATTCCA	12	2	0.001	*CG5288*	Galactose kinase	5288	66E6
TTAAAATTAA	8	0	0.001	*CG15425*	Unknown	15425	24E1
TTTAGTTCTT	10	1	0.001	*RH08983.3′*	–	–	33E9
TACAGCGTAA	13	3	0.002	*Peb*[b]	Testis-specific transcript	2665	60F5
TACTTATGTC	9	1	0.002	*CG3488*	Esterase/lipase/thioesterase family	3488	23D4
ACGTCAGAAC	12	3	0.003	*BcDNA:LD06023*	Unknown	–	102F4
GTTCTTTTTC	10	2	0.005	*CG2736*	Scavenger receptor, defense response	2736	60F4
TGAGGATGAA	10	2	0.005	*Actn*	Calcium binding, cytoskeletal anchor protein	4376	2C4–7
TTTGAACATT	10	2	0.005	AE003804/AE003542	–	–	53E10–54B8/68F1–69A3
AAAACAACAG	8	1	0.006	AE003803	–	–	54B8-C11
ACGATGTTGA	6	0	0.006	*CG1764*	Dimethylargininase	1764	11E9
ATGATTACCA	6	0	0.006	*CG5344*	RAB GTPase activator	5344	86E15
ATGGTATGCC	8	1	0.006	*CG17661*	Ligand binding or carrier, calcium binding	17661	Unknown
CGGATTGCTG	6	0	0.006	AE003677/AE003736	–	–	84E4-F2/93F3–F12
CTGAAACATC	8	1	0.006	*GM06073.5′*	–	–	67E1–3
GACCCTAACC	6	0	0.006	*Mio*	Transcription factor	18362	39C1–3
GCGGCAGTGG	8	1	0.006	*CG11236*	D-aspartate oxidase	11236	27B1
TATTGAATAT	8	1	0.006	*DIP1*	dsRNA binding, chromatin binding	17686	20A
TTAGAATGGT	8	1	0.006	*HL04936.5′*	–	–	82C3
TTTGGAATAA	6	0	0.006	*CG2131*	Procollagen *N*-endopeptidase,	2131	39F1–3
TTTTCCAATT	8	1	0.006	*CG12012*	Unknown	12012	63D2
ACCAAGGCAC	0	9	0.007	*CG7461*	Very long-chain acyl-CoA dehydrogenase	7461	56D3
CGAGCAGAAA	0	9	0.007	AE003649	–	–	35D7–F1
CTGTCAACCT	0	9	0.007	*fau*	Anoxia up-regulated protein	6544	86C4
ACTTTTATGT	1	12	0.008	*RH19272.5′*	–	–	24A5
ACAATATACG	9	2	0.009	*CG30069*	Unknown	30069	50E8
GATCGCTACC	9	2	0.009	*msta*	Unknown	18033	2E2
TATATCTATA	9	2	0.009	*cmp44E*	Plasma membrane protein	8739	44E4
TATTACTGCC	9	2	0.009	*CG9645*	Enteropeptidase	9645	88A12
TTCTTGAATG	9	2	0.009	*CG11572*	Unknown	11572	102A7–8

Male tag numbers are normalized to female tag numbers (coefficient is 1.4). Tags are listed as $P < 0.01$, and fold difference $\geqslant 4$. *P* was calculated using Monte Carlo Simulation in the SAGE 2000 Software v4.12. Gene symbol, predicted function, CG number and location are retrieved from FlyBase at http://flybase.bio.indiana.edu. If a tag matches multiple ESTs from the same gene, only one EST is listed. Due to biological (alternative splicing, multiple sites of polyadenylation) and technical (incomplete digestion of cDNAs with *Nla*III) reasons, several tags may be annotated to a single gene. In these cases, only the most abundant tag is listed in this table. Genes known previously to be expressed in a sex-biased manner are in bold.

[a]Northern blot analyses for these tags are shown in Figure 2B.

[b]By Northern blot analysis, these tags were found to be expressed only in the male abdomen, but not the head (data not shown).

determination cascade should be expressed in ψ-males and, conversely, female-specific genes should be repressed. We first analyzed expression of *tsx*, *sxe1* and *sxe2* in heterozygous males and females of these various strains, which confirmed that these genes were only expressed in males but not females (Figure 3C; data not shown). We then investigated the *tsx*, *sxe1* and *sxe2* expression in XX flies homozygous mutant for *Sxl*, *tra* and *tra2*, and found that all were expressed in these ψ-males (Figure 4A and B). Expression levels for *tsx* were much higher in *tra2* ψ-males and *X/B Y;CyO/tra2* males than *tra* ψ-males or wild-type males, suggesting that increased expression is caused by the genomic background in the *CyO/tra2* strain, but not the *tra2* mutation itself. In any case, there was no significant difference in expression levels of all three genes between XY males and XX ψ-males within a given strain (Figure 4A, compare lanes 3 and 5; B, compare lanes 3 and 4, and 5 and 6), indicating that *tsx*, *sxe1* and *sxe2* are regulated by *Sxl*, *tra* and *tra2*. These results are also consistent with the RNA *in situ* hybridization experiments, which showed *tsx* expression in *tra2* ψ-males (Figure 3A).

The dominant Y-linked eye mutation *Bar*^{stone} (*B*^{s}) is present in the *tra* and *tra2* strains to distinguish sexually transformed XX ψ-males from XY males. *B*^{s} reduces the size of the eye and the visual brain centers by ~90%

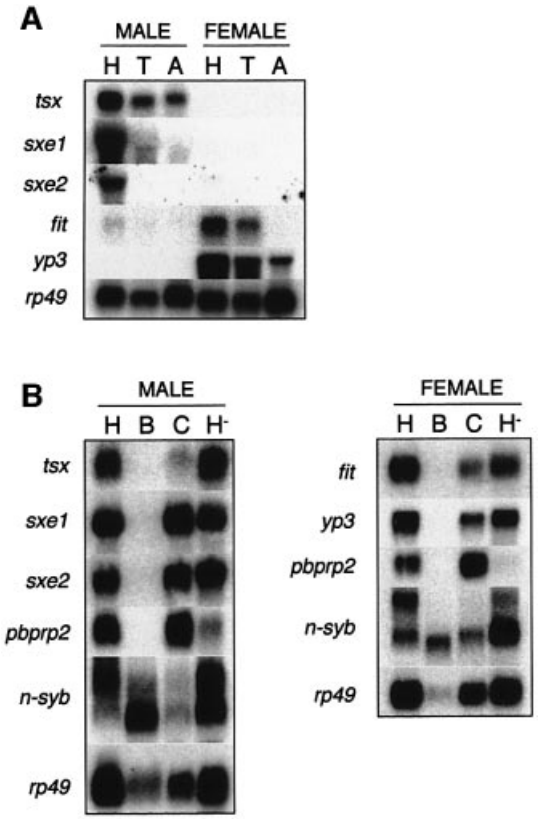


Fig. 2. Expression of *tsx*, *sxe1*, *sxe2* and *fit* is restricted mainly to the head. (**A**) Northern blot analysis was carried out with RNA isolated from head (H), thorax (T) and abdomen (A) to determine sex- and tissue-specific expression. Approximately 5 μg of total RNA were loaded in each lane. Identical northern blots were hybridized with probes for *tsx*, *sxe1*, *sxe2* and *fit*. An *rp49* probe was used as an RNA loading control. (**B**) Northern blot analysis of various head tissues from males and females: brains without the optic lobes (B) were dissected from the remaining parts of the head carcass (C), which consisted mainly of the cuticle (containing no RNA) and the fat cells, as well as the visual, olfactory (antenna and maxillary palps) and taste sensory systems (labellum). Note that deep fat cells in the head were partly lost in the dissection procedure and are therefore reduced in either B or C. The H^- lane contained RNA isolated from heads in which all chemosensory organs (antenna, maxillary palps and labellum) were removed. Identical northern blots were hybridized with probes for *tsx*, *sxe1*, *sxe2* and control genes (*pbprp2*, *n-syb* and *rp49*) or for *fit* and control genes (*yp3*, *pbprp2*, *n-syb* and *rp49*), respectively.

(Childress, 1973). As shown in Figure 4B, males with wild-type and B^s eyes have similar transcript levels of all three male-specific genes (compare lanes 1 and 3), indicating that the visual system cannot be the major site of expression for *tsx*, *sxe1* and *sxe2*. This observation is consistent with previous northern blots and *in situ* hybridization experiments (Figures 2B, 3A and B).

To test whether *tsx*, *sxe1* and *sxe2* were indeed dependent on *dsx*, we investigated their expression in flies with different *dsx* mutations. dsx^1 is a null mutation, generating no functional DSX protein (Nöthiger *et al*., 1987; Burtis, 1993). Thus, dsx^1 homozygous mutant flies are intersexual in phenotype, regardless of their karyotype. dsx^D is an insertion of a transposable DNA element in the fourth, female-specific exon, leading to TRA-independent, male-specific *dsx* splicing (Nagoshi and Baker, 1990). Therefore, in hemizygous *X/X*; $dsx^D/Df[dsx]$ flies, only DSX^m but no DSX^f is produced, whereas in heterozygous *X/X*; dsx^D/dsx^+ flies, both forms of DSX are generated (DSX^f from the dsx^+ allele and DSX^m from the dsx^D allele). Northern blot analysis of RNA isolated from heads of these various *dsx* mutant flies indicated that all three genes are controlled by *dsx* (Figure 4C). First, *tsx*, *sxe1* and *sxe2* were expressed, albeit at reduced levels, in dsx^1 homozygous XX and XY intersexes, suggesting that the absence of functional DSX leads to basal expression of these genes (Figure 4C, lanes 3 and 4). Secondly, *X/X*; dsx^D/Df ψ-males (producing only DSX^m) and *X/X*; dsx^D/dsx^+ intersexes (producing both DSX^m and DSX^f) also express all three genes (Figure 4C, lanes 5 and 6). Thus, our data demonstrate that *tsx*, *sxe1* and *sxe2* are downstream effectors of the sex determination cascade and may be direct targets of *dsx*.

fit is dependent on Sxl, but not on tra and tra2

As for the male-specific genes, we investigated the regulation of the female-specific gene *fit* using flies mutant for the sex determination genes. Genes normally expressed only in females are expected to be repressed in any ψ-male, if regulated by the sex determination cascade. We found that *fit* expression was indeed abolished in *Sxl* ψ-males (Figure 5A, lane 5). *Sxl/Y* males showed no *fit* expression, just like wild-type males, because males do not produce any SXL protein (Figure 5A, lane 3). This result suggested that *fit* expression is dependent on *Sxl* function, just as observed for the three male-specific genes.

We then asked whether *fit* is also dependent on regulators downstream of *Sxl*. Northern blot analysis of homozygous mutant *tra* and *tra2* flies revealed that ψ-males showed high levels of *fit* expression that typically are observed in heterozygous females of these strains (Figures 5B, lanes 4 and 6, and 3C, lane 6; data not shown). Similarly, homozygous males showed a very low level of *fit* expression, similar to that observed for heterozygous XY males of these strains (Figures 5B, lanes 3 and 5, and 3C, lane 5; data not shown). Taken together, these data indicate that *fit* expression is independent of *tra*, *tra2* and, hence, the downstream regulators *dsx* and *fru*. In addition, analysis of RNA from different strains revealed rather large variability in expression levels of *fit* in females. In strains with elevated levels of female *fit* expression, low levels were observed in males (Figures 3C and 5B).

Ectopic expression of TSX in females reduces receptivity

To investigate a potential role for TSX in behavior, we constructed GAL4-dependent UAS reporters with epitope-tagged (V5; see Materials and methods) cDNAs for these genes and generated transgenic flies. The TSX protein was stably expressed under the control of a ubiquitously expressed GAL4 driver (*actin:GAL4*; data not shown). We tested whether ectopic expression of TSX caused any effects on mating behavior and measured mating performance of males and females by determining latency time in single mating set-ups (for details, see Materials and methods). No obvious effects on mating performance were observed in males with ubiquitous TSX expression, but we observed that TSX-expressing females were negatively affected (Table II). In both lines tested,

significantly fewer TSX-expressing females mated during the observation period (15 min) when compared with control females. The increase in non-maters is probably caused by an elevated escape response, as TSX-expressing females often run away from an approaching male. Taken together, our experiments suggest that ectopic TSX expression interferes with normal female mating response.

Discussion

Genetic investigations have led to an understanding of the molecular splicing cascade that culminates in the expression of sex-specific transcription factors encoded by *dsx* and *fru*. We hypothesized that a detailed analysis of gene expression differences between adult males and females

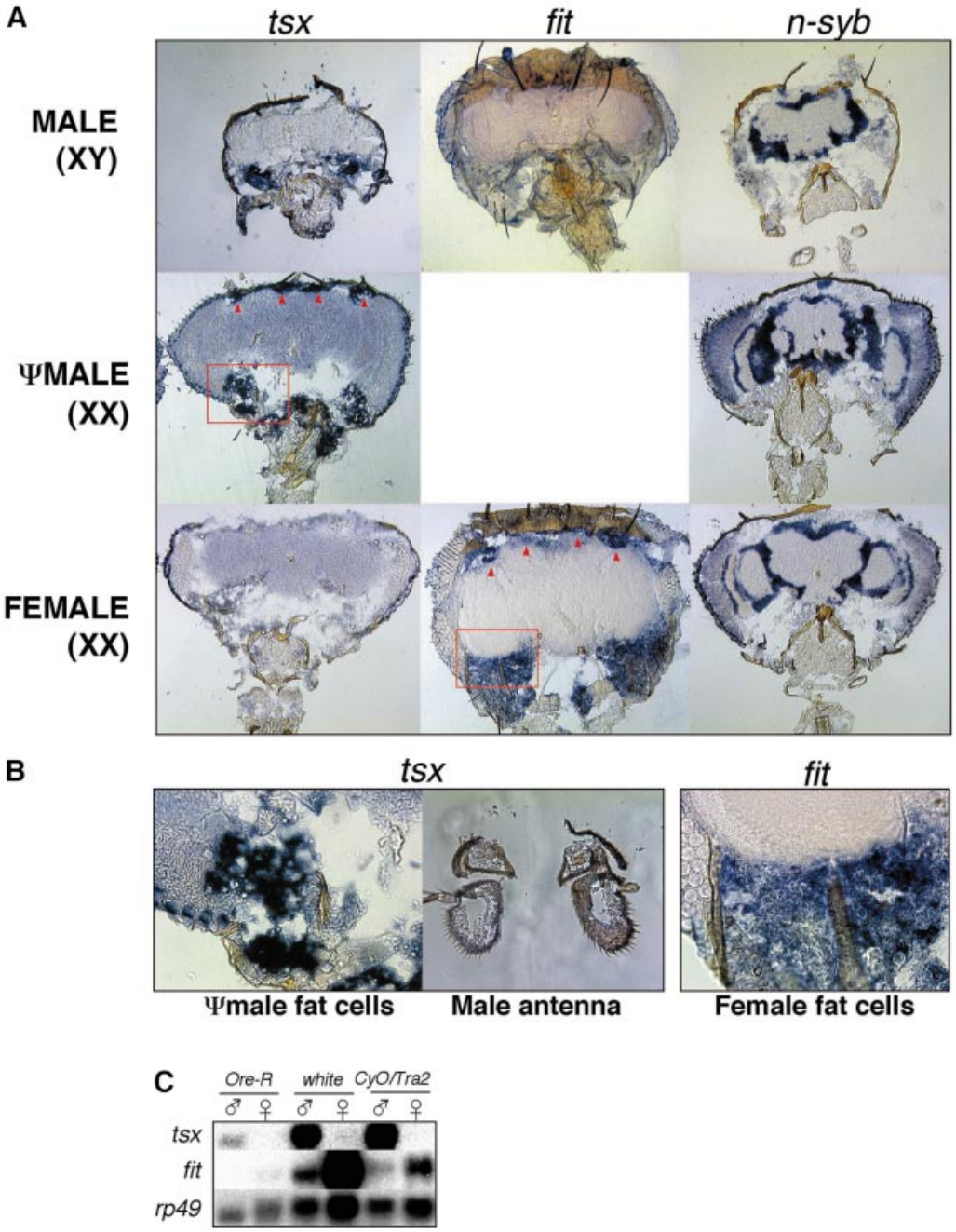


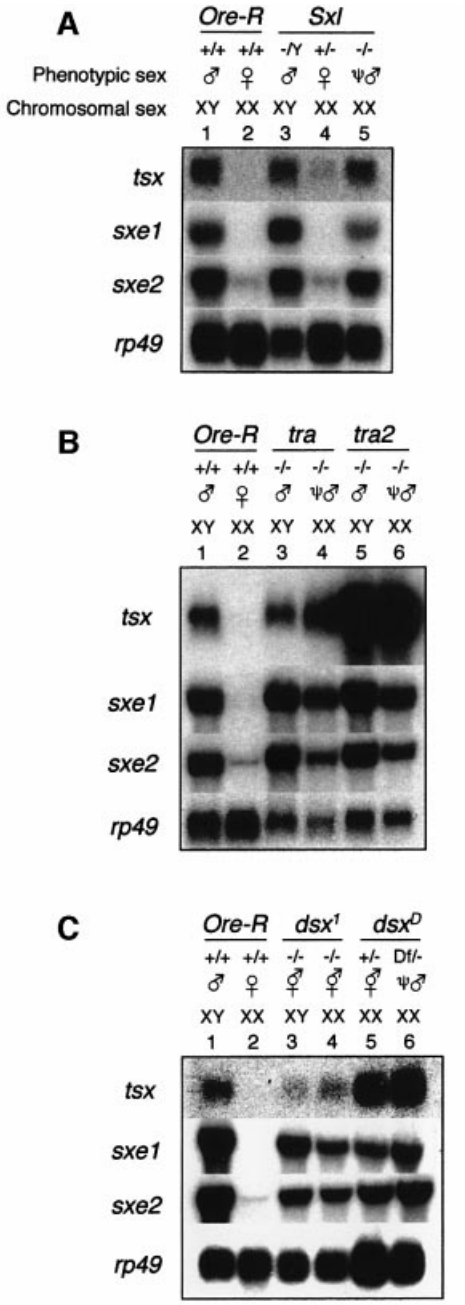
Fig. 3. *tsx* and *fit* are expressed in the fat cells of the head. (**A**) *In situ* hybridization of digoxigenin-labeled antisense RNA derived from a *tsx* and *fit* cDNA clone reveals specific signals in large fat cells in the ventral and dorsal region (red arrows) of the head capsule of *CyO* males, *tra2* ψ-males (*tsx*) and w^{1118} females (*fit*), respectively. Note the absence of signal in the brain and the visual system. The female (*tsx*) and the male (*fit*) head show no hybridization signals. Sections of males, ψ-males and females were also hybridized with antisense RNA probes derived from *n-syb* (right panel) to indicate the location of neuron cell bodies in the brain. Strains used were B^sY; *CyO/tra2* (*tsx*; *n-syb*) and w^{1118} (*fit*). (**B**) Enlargement of the ventral region of the head containing a large number of deep fat cells located below the brain (first and third frame; see red rectangles in A). Also note the absence of *tsx* hybridization signals in the antenna (middle frame). (**C**) Northern blot of males and females of the three strains used for the *in situ* hybridization experiment. Note the several fold higher levels of *fit* and *tsx* RNA in both w^{1118} and *CyO/tra2* strains when compared with *Ore-R*. Also note that low *fit* expression can be observed in males of these strains.

could lead to an understanding of the critical sex-specific physiological events downstream of these regulators. The characterization of the first few candidates identified by SAGE has established the value of this approach.

fit is regulated by a *tra*-independent pathway

An unexpected result was the identification of a *Sxl*-dependent, but *tra*- and *tra2*-independent pathway that mediates female-specific *fit* expression. It is generally believed that most aspects of sexual differentiation and behavior are mediated by *tra* and *tra2* (Cline and Meyer, 1996; Baker *et al*., 2001). However, this assumption has not been tested rigorously for female mating behaviors.

How is *fit* expression controlled in females? First, *fit* could be regulated directly by SXL, which seems unlikely, because SXL functions post-transcriptionally (Bell *et al*., 1988, 1991; Salz *et al*., 1989; Valcarcel *et al*., 1993; Bashaw and Baker, 1995). The known RNA targets of SXL, *Sxl*, *tra* and *msl2* pre-mRNAs, are stably expressed in the absence of SXL in males (McKeown *et al*., 1987; Bell *et al*., 1988; Bashaw and Baker, 1995; Kelley *et al*., 1995; Zhou *et al*., 1995). Therefore, if *fit* RNA were a target of SXL, we would expect to find stable *fit* RNA precursor in males as well as conserved SXL-binding sites within the *fit* pre-mRNA. Neither is the case for *fit*. A second possibility is that *fit* is controlled through a *Sxl*-dependent, unknown regulator. We identified several strains in which females expressed higher levels of *fit* RNA than wild-type *Ore-R* females (Figure 3C). Variations in *fit* expression are probably caused by differences in genetic backgrounds, which could include both *cis*-regulatory elements in the *fit* promoter and *trans*-acting factors. Identifying these elements might provide an opportunity to elucidate the nature of the *tra*-independent pathway that regulates *fit* expression. Naturally, exogenous factors, such as temperature, diet, population density, etc., may also influence *fit* expression.



Sex-specific functions may be mediated by the fat cells in the head

Another intriguing result from our investigations is the observation that none of the four genes is expressed at significant levels in the brain. Instead, our studies show that *tsx*, *sxe1*, *sxe2* and *fit* are expressed mainly in the fat cells of the head (Figures 2 and 3), which suggests that these genes create different physiological conditions in the adult male and female head important for sex-specific functions.

We propose that *fit*, *sxe1* and *sxe2*, which are expressed mainly in the head, may exert their functions on the brain. Similar to the pituitary in the head of mammals, the fat cells could play the role of an endocrine organ. Such a function has been shown for the fat cells of females, where the YPs are synthesized and released into the hemolymph (Butterworth *et al*., 1991, 1999).

Based on the predicted protein sequences, we can envisage sex-specific roles for *sxe1* and *tsx*. *tsx* encodes a

Fig. 4. Expression of *tsx*, *sxe1* and *sxe2* is dependent on *Sxl*, *tra* and *dsx*. Blots were hybridized with RNA from manually dissected heads. Approximately 5 μg of RNA were loaded per lane. All blots were re-hybridized with an *rp49* probe to monitor RNA loading. Head RNA from *Ore-R* males and females was used for comparison in each experiment (lanes 1 and 2). (**A**) Expression of *tsx*, *sxe1* and *sxe2* in *Sxl* mutant flies: *Sxl* Ψ-males express all three male-specific genes, as indicated by strong hybridization signals in lane 5. No expression was observed in heterozygous *Sxl* females (lane 4). Genotypes were: *y cm* Sxl^{f7M1}*ct v/Y* males (lane 3); *w* Sxl^{M1f3}*sn/Binsinscy* females (lane 4); and *y cm* Sxl^{f7M1}*ct v/w* Sxl^{M1f3}*sn* Ψ-males (lane 5). (**B**) Expression of *tsx*, *sxe1* and *sxe2* in *tra* and *tra2* mutant flies: both *tra* and *tra2* Ψ-males strongly express all three male-specific genes, as indicated by the strong hybridization signals in lanes 4 and 6, respectively. Genotypes were: *X/*B^sY; *th st tra cp ri* p^p*/tra e ca* males (lane 3); *X/X*; *th st tra cp ri* p^p*/tra e ca* Ψ-males (lane 4); B^sY; *cn tra2 bw/cn tra2 bw* males (lane 5); and *X/X*; *cn tra2 bw/ cn tra2 bw* Ψ-males (lane 6). (**C**) Expression of *tsx*, *sxe1* and *sxe2* in various *dsx* mutant flies: XX flies with various *dsx* mutations show a low to intermediate level of expression of *tsx*, *sxe1* and *sxe2*. Note the dramatic increase of *tsx* expression in flies expressing DSX^m (lanes 5 and 6) when compared with flies with no functional DSX protein, suggesting an activating role for *dsx* on *tsx*. Genotypes were: *X/*B^sY; dsx^1/dsx^1 intersexes (lane 3); *X/X*; dsx^1/dsx^1 intersexes (lane 4); *X/X; TM6/*dsx^D intersexes; and *X/X*; *Df[dsx]/*dsx^D Ψ-males; *X/X; TM6/*dsx^D intersexes (lane 5); and *X/X*; *Df[dsx]/*dsx^D Ψ-males (lane 6).

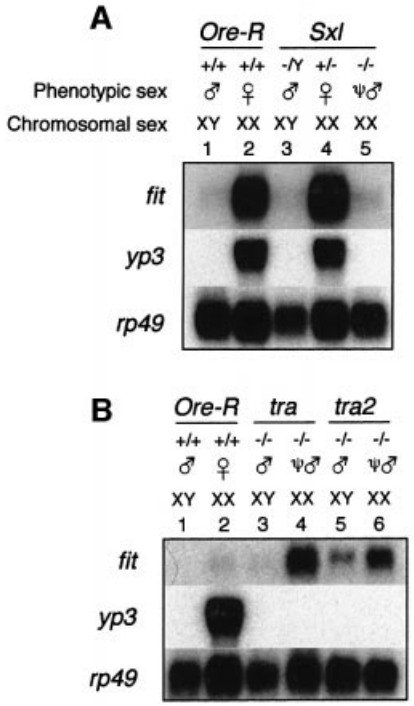


Fig. 5. *fit* is not regulated by *tra* and *tra2*. Blots were hybridized with RNA from manually dissected heads. Approximately 5 μg of RNA were loaded per lane. All blots were re-hybridized with an *rp49* probe to monitor RNA loading. RNA from *Ore-R* males and females was used for comparison in each experiment (lanes 1 and 2). (**A**) Expression of *fit* in *Sxl* mutant flies. *Sxl* ψ-males do not express the *fit* gene, as indicated by complete lack of signal in lane 5. Note that *yp3* is also dependent on SLX. Genotypes were: *y cm* $Sxl^{f7,M1}$*ct v/Y* males (lane 3); *w* $Sxl^{M1,f3}$*sn /Binsinscy* females (lane 4); and *y cm* $Sxl^{f7,M1}$*ct v/w* $Sxl^{M1,f3}$*sn* ψ-males (lane 5). (**B**) Expression of *fit* in *tra* and *tra2* mutant flies. Both *tra* and *tra2* ψ-males express significant levels of *fit* RNA, similar to that observed in heterozygous females of these strains (see Figure 3C), as indicated by clear hybridization signals in lanes 4 and 6, respectively. A very low level of *fit* expression was observed in both homozygous XY males (lanes 3 and 5) and heterozygous males (Figure 3C, lane 5). Genotypes were *X/*B^sY; *th st tra cp ri* p^p*/ tra e ca* males (lane 3); *X/X*; *th st tra cp ri* p^p*/ tra e ca* ψ-males (lane 4); B^sY; *cn tra2 bw/cn tra2 bw* males (lane 5); and *X/X*; *cn tra2 bw/ cn tra2 bw* ψ-males (lane 6).

member of the *opbp* gene family. Oderant/pheromone binding proteins (OPBPs) are expressed generally in support cells of chemosensory sensilla and secreted into the extracellular lymph space, where they interact with odor and taste ligands to increase their solubility, protect them from degradation or remove them from the lymph space (Pikielny *et al*., 1994; Galindo and Smith, 2001). We suggest that TSX has been co-opted for a role to interact with and transport small molecules in the head. Upon release from the fat cells, TSX bound to a ligand may reach a target organ, for example the brain, to exert its physiological effects. The reduced mating activity of females ectopically expressing TSX is consistent with such a role (Table II). In addition, a precedent for a protein related to *Drosophila* OPBPs with a putative function unrelated to chemosensation has been reported in rats (Schoentgen and Jolles, 1995).

Expression of all *Drosophila opbp* genes, including *tsx*, has been analyzed previously using the GAL4/UAS system (Galindo and Smith, 2001). These experiments

Table II. Reduced mating performance of females ectopically expressing TSX protein

Genotype	*n*	Fraction of females that fail to copulate (%)	Latencies (min)
Act:GAL4/UAS:tsx_V5.4	60	**41.7**	5.9 ± 0.7
UAS:tsx_V5.4/+	64	19.4	5.6 ± 0.6
Act:GAL4/UAS:tsx_V5.5	60	**26.7**	5.7 ± 0.6
UAS:tsx_V5.5 /+	55	10.9	5.8 ± 0.5
Act:GAL4/+	63	15.9	5.8 ± 0.6
White	63	12.7	5.5 ± 0.5
Wild type	63	15.9	3.8 ± 0.6

Individual virgin females (3–6 days old) of the genotype indicated were placed with a single *Ore-R* male (3–8 days old) in a mating chamber (see Materials and methods). Each pair was observed for 15 min. The time to copulation (latency) was recorded and averaged. The mean latencies and SEMs were calculated only for pairs that mated within the 15 min observation period. Note that the fraction of females that fail to copulate within the 15 min observation period is significantly larger in the experimental females (genotypes in bold) than in the various controls.

revealed *tsx* expression in chemosensory organs of both adult sexes. In contrast, our northern blot analysis and RNA *in situ* hybridization experiments showed that *tsx* transcripts in the adult are male specific and found mainly in the fat cells of the head, but not the chemosensory organs (Figures 2B and 3). Moreover, we observed abundant non-sex-specific expression in the larvae and pupae. The most likely explanation for these discordant results is the lack of multiple, essential regulatory promoter elements in the GAL4 driver used in the previous study (Galindo and Smith, 2001), which would therefore inaccurately represent *tsx* expression. However, our studies do not exclude the possibility of low *tsx* expression in the chemosensory system, which would be consistent with a role in pheromone detection. Such a role is appealing, because *Drosophila* mating behavior is mediated by taste cues recognized by sensory bristles located in the labellum and the forelegs (Nayak and Singh, 1983). In addition, strain-specific differences might also account for the non-sex-specific expression observed in the *tsx:Gal4* driver.

The second gene for which we wish to propose a function, *sxe1*, encodes a cytochrome P450 protein (CYP), members of which have been studied extensively in mammals and insects. One major role of these enzymes is liver detoxification, whereby toxic, water-insoluble metabolites are rendered sufficiently water soluble to be excreted in the urine. A second, important function for CYPs is their role in steroid hormone metabolism, in both mammals and insects. Of particular interest in this regard is cytochrome P450arom (CYP19), which has been widely implicated in sex-specific functions in vertebrates (Roselli and Resko, 1997; Fisher *et al*., 1998; Conley and Hinshelwood, 2001).

In insects, CYPs are involved in ecdysone metabolism, specifically in hydroxylation of cholesterol precursors (Mayer *et al*., 1978; Smith *et al*., 1979; Grieneisen *et al*., 1993; Adams *et al*., 2000). *Disembodied* (*dib*), the only studied *Cyp* gene in *Drosophila*, is involved in ecdysone metabolism during embryogenesis (Chavez *et al*., 2000). However, ecdysone triggers most transition phases during development, including larval molts and various differen-

tiation processes in the pupae during metamorphosis (Mitsui and Riddiford, 1978; Riddiford, 1993). A reported function of ecdysone in the adult is its involvement in *yp* gene regulation in the fat body of females (Shirk *et al*., 1983).

The expression of *sxe1* in the fat cells of the male head suggests the intriguing possibility that small molecules (e.g. steroid hormones) might be synthesized in a sex-specific fashion. Released into the circulatory system, they could reach any organ in the adult male fly, including the brain, and hence mediate sex-specific physiological states that could affect behaviors. One target of such a male-specific hormone might be the neurons in the brain expressing DSF, an orphan nuclear hormone receptor, which controls different male- and female-specific behaviors in adult flies (Finley *et al*., 1997, 1998).

A role for sex-specific genes in behavior

Expression and regulation of the genes reported here provide a means to generate distinct physiological states in the head of the two sexes. What are the likely consequences of these differences? What aspects of maleness and femaleness are affected by the presence/absence of these proteins and enzymes? It is tempting to speculate that these genes play specific roles in sexual behaviors, simply because of their implicated biochemical function (i.e. *tsx* and *sxe1*). However, our knowledge about the extent of behavioral differences between sexes is limited by the way we study fruit flies, namely in confined spaces with abundant food sources and mating choices. For example, very little is known about foraging, energy consumption, food intake, grooming, locomotion, etc. in wild fruit flies in general, and with regard to potential differences between the sexes in particular. Thus, some of the sex-specific genes described here may be involved in aspects of physiology and behavior not related to courtship and mating. In this regard, it is interesting to note that two of the eight genes, *sxe1* and *sodh-1* (Figure 1; Table I), were also isolated in a microarray analysis for genes under circadian control, and *tsx* was found in the same study to be under the control of the circadian regulator CLK (McDonald and Rosbash, 2001). Moreover, recent behavioral studies reported sex-specific differences in locomotor activity between males and females (Helfrich-Forster *et al*., 2001). Thus, these observations suggest that sex-specific physiological differences in adult *Drosophila* may influence a variety of behaviors not linked to courtship.

Materials and methods

Fly strains and genetics

The following fly strains were used: *Ore-R, w^{1118}, w $Sxl^{M1,f3}$sn/$\hat{Y}X$ y f, y cm Sxl^{f7M1}, ct v/Binsinscy, B^sY; th st tra cp ri p^p/TM3, tra e ca/TM6, B^sY; cn tra2 bw/CyO, Df [dsx]/TM3, B^sY; dsx^1/TM3, B^sY; hs→tra^f Df[3L] st tra p^p dsx/dsx^D Sb e/TM6*. Crosses were performed at 25°C. For the generation of SAGE libraries, we used manually dissected male or female heads of 2- to 4-day-old Ore-R flies.

SAGE

SAGE libraries were generated as described in 'Serial Analysis of Gene Expression', version 1.0d with minor modifications. The protocol and analysis software, SAGE 2000 Software Version 4.12, were obtained from The Johns Hopkins University (see also http://www.sagenet.org). Poly(A)$^+$ RNA was isolated from collected heads with RNAzol B (TEL-TEST, Inc.) and oligo(dT) columns (Stratagene). Dynabeads oligo(dT)25 (Dynal) were used as primer to synthesize cDNA from 1.0 μg of poly(A)$^+$ RNA. Double-stranded cDNA was digested with *Nla*III (NEB) and collected by a magnet. After linker ligation, DNA fragments were digested with the tagging enzyme, *Bsm*FI (NEB), and ligated. PCR amplification (26 cycles) was carried out with biotinylated primers and 1% of the ligation products as template. After *Nla*III (NEB) digestion of the 102 bp PCR products, the released 26 bp fragments were purified by the streptavidin-linked magnetic beads and PAGE. After 1.5 h concatenation, concatemers were cloned into the *Sph*I site of pZero-1 (Invitrogen). Purified plasmids from cultured transformants were screened for long inserts (>800 bp) by *Xba*I and *Xho*I digestion. We sequenced 2236 (screened 8640) and 2496 (screened 10 848) plasmids in the male and female head libraries, respectively. Sequencing was performed on the ABI 3700 automated DNA sequencer (Applied Biosystems).

Analysis and annotation of SAGE tags

Sequence data were analyzed using SAGE 2000 Software Version 4.12. To build the database for annotation, we downloaded data sets from The Berkeley *Drosophila* Genome Project (BDGP; http://www.fruitfly.org) on November 23, 2001 as the following: na_geno.dros.RELEASE2 (The Celera/BDGP whole-genome shotgun sequence from Release 2), na_gadfly.dros.RELEASE2 (nucleic acid sequence for every predicted transcript cDNA from Release 2), na_EST.dros [*Drosophila* expressed sequence tag (EST) sequences from BDGP], na_cDNA.dros (*Drosophila* full-length cDNA sequences from BDGP) and na_gb.dros [*Drosophila* sequences from GenBank (minus BDGP and EDGP sequences)]. Using the 'Genomic mode' of SAGE 2000 Software, we extracted all the 10 bp sequences downstream of the CATG site. We annotated tags to genes using the following criteria: (i) tags matched to the 3′-most CATG site of the full-length cDNA or 3′ EST; (ii) tags matched to the 3′-most CATG site of the predicted gene; and (iii) tags matched to only genomic DNA sequence and the matched CATG site is located within 2 kb downstream of the predicted gene.

Northern blot analysis

Total RNA was used for all northern blot analysis in this report. RNA was loaded on formamide–agarose gels (1%) and blotted to Hybond-N+ membranes (Amersham/Pharmacia). In Figure 1B, oligo DNA and PCR probes were used. The StarFire oligo DNAs (50 bases) were labeled with [^{32}P]dATP using the StarFire DNA Labeling System (Integrated DNA Technologies). The names of probes and their sequences are the following:

StF-*Cyp4d21* (5′-TTAAGTTTGCTCCTGGCATGTAACATAAAATAAATACAAAAAAATGTAAA-3′);

StF-*yellow-c* (5′-TAATGTCATAATGTAAATACAAAAATGCACCGCGTCATAAACACAGCATG-3′);

StF-*CG4979* (5′-CACCGCCCATGAACTCGTTGCCGGTGCATGTATTGACTGCATATTTTTCG-3′);

StF-*CG17820* (5′-TGAATGAATGACATGGTGTATCCAATCGAACGCCTAACGCAGTGCACGTC-3′). Other probes (coding region of each genes) were labeled with [^{32}P]dCTP by random priming. The cDNA clones for *Sodh-1* (LP12301, gb;AI297864), *CG7433* (GM13560, gb;AA803585), *CG7592* (LP05187, gb;AI261107) and *CG11458* (GH15115, gb;AI238960) were kindly provided by Dr Todd Laverty.

RNA in situ hybridization

RNA *in situ* hybridization was carried out essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993). This protocol was modified to include detergents in most steps, thereby increasing sensitivity and reducing background. The hybridization buffer contained 50% formamide, 5× SSC, 5× Denhardt's, 250 μg/ml yeast tRNA, 500 μg/ml herring sperm DNA, 50 μg/ml heparin, 2.5 mM EDTA, 0.1% Tween-20 and 0.25% CHAPS. Anti-DIG antibody (Boehringer Mannheim) steps were in the presence of 0.1% Triton X-100, and the reaction was developed for ~18 h in buffer containing 0.1% Tween-20 using the NBT/BCIP kit from Promega. Slides were mounted in Glycergel (Dako) and viewed with Nomarski optics.

Tissue dissections

Flies were anesthetized with CO_2, decapitated and placed in a depression well dish in 1× phosphate-buffered saline (PBS) at room temperature. Brains were dissected out using fine forceps (no. 4 or finer) and placed directly into a microcentrifuge tube with 0.5 ml of ice-cold 1× PBS. The remaining carcasses were gathered into a separate tube and also placed on ice. When finished with all flies, tubes were quickly (~10 s) spun down on a tabletop picofuge and PBS removed. Parts were immediately frozen on

dry ice and then stored at –80°C until RNA was prepared. For the H^- fraction, labella and antennae were carefully removed from heads using forceps and then processed in the same manner as above.

Cloning of expression constructs and generation of transgenic flies

A cDNA of the *tsx* gene was obtained by RT–PCR from male head RNA and cloned into the vector pET-DEST42 (Invitrogen). The V5-tagged TSX fusion gene was then cloned into the pUAST vector (Brand and Perrimon, 1993) and transgenic flies were generated according to Amrein and Axel (1997).

Mating assay

Males and females in mating assays were collected within 8 h after hatching and reared on standard food in isolation. They were kept on a 12 h light:12 h dark cycle for 3–8 days. Matings were performed in mating chambers (small plastic cuvettes) and were observed until copulation occurred, but not longer than 15 min. The genotype of the experimental males was generated from the following crosses: *Act:GAL4/UAS-tsx_V5.4* (or *V5.5*)*: yw/w*; *act:GAL4/TM3SerGFP* × *w*; *UAS:tsx_V5.4* (or *V5.5*); *UAS:tsx_V5.4* (or *V5.5*)*/+: OreR* × *w*; *UAS:tsx_V5.4* (or *V5.5*); *Act:GAL4/+: OreR* × *yw/w*; *act:GAL4/TM3SerGFP*.

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