A genomic analysis of *Drosophila* somatic sexual differentiation and its regulation

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Summary

In virtually all animals, males and females are morphologically, physiologically and behaviorally distinct. Using cDNA microarrays representing one-third of *Drosophila* genes to identify genes expressed sex-differentially in somatic tissues, we performed an expression analysis on adult males and females that: (1) were wild type; (2) lacked a germline; or (3) were mutant for sex-determination regulatory genes. Statistical analysis identified 63 genes sex-differentially expressed in the soma, 20 of which have been confirmed by RNA blots thus far. In situ hybridization experiments with 11 of these genes showed they were sex-differentially expressed only in internal genital organs. The nature of the products these genes encode provides insight into the molecular physiology of these reproductive tissues. Analysis of the regulation of these genes revealed that their adult expression patterns are specified by the sex hierarchy during development, and that *doublesex* probably functions in diverse ways to set their activities.

Key words: *Drosophila*, Sex determination, Microarray, Somatic, Reproduction

Introduction

In essence, sexual reproduction is the process whereby two gametes, one contributed by each parent, fuse to form a new individual. Achieving this end is an elaborate process that in multicellular animals requires, along with germline development, the appropriate sex-specific development and physiology of the external genitalia, portions of the nervous system that control sex-specific reproductive behaviors, somatic tissues of the gonads (which play important roles in gametogenesis), and the internal genital organs (whose products are important both pre- and post-copulation for successful reproduction). Currently, we have limited knowledge, in any organism, of the sets of genes that are deployed sex-differentially in adult somatic tissues, and limited knowledge of their roles in sexual reproduction.

Drosophila melanogaster is a powerful model system in which to acquire an understanding of the sex-specific physiology of adult somatic tissues, because we have a thorough understanding at the molecular-genetic level of the regulatory hierarchy that controls somatic sexual differentiation (Fig. 1) (reviewed by Cline and Meyer, 1996; Baker et al., 2001; Christiansen et al., 2002). There have been significant advances in understanding how the actions of DSX^F and DSX^M, terminal transcription factors in the hierarchy encoded by the *doublesex* (*dsx*) gene, are integrated with other key developmental hierarchies to achieve sex-specific patterns of growth, morphogenesis and differentiation (reviewed by Christiansen et al., 2002). However, we have relatively little knowledge of the genes that are sex-differentially deployed in adults through the action of the two final genes in the hierarchy, *dsx* and *fruitless* (*fru*), which encodes (among several isoforms) a male-specific transcription factor hereafter referred to as FRU^M.

Several approaches have been used to identify genes expressed sex-differentially in *D. melanogaster* adults. The most thoroughly studied tissue is the male accessory gland, in which 75 genes have been identified using biochemical purification and differential cDNA hybridization (reviewed by Wolfner, 2002). Several of these genes encode proteins whose effects in the mated female have been characterized and include decreasing female receptivity to re-mating, increasing ovulation and egg laying, and facilitating sperm storage. Additional screens have focused on sex-differential gene expression in the head and foreleg. In head tissues, subtractive hybridization identified *takeout* (Dauwalder et al., 2002), and serial analysis of gene expression (SAGE) uncovered 46 sex-differentially expressed genes (Fujii and Amrein, 2002). From the foreleg, two genes implicated in male-specific chemosensory function (*CheA29a* and *CheB42a*) were isolated by subtractive cloning (Xu et al., 2002). Sex-differential gene expression in adults has also been studied using microarray technology (Jin et al., 2001; Arbeitman et al., 2002; Parisi et al., 2003; Ranz et al., 2003). In two of these studies (Arbeitman et al., 2002; Parisi et al., 2003), both the somatic and germline

components of sex-differential expression were determined, but regulation by the sex-determination hierarchy was not explored.

Here, we identify genes that are expressed sex-differentially in somatic tissues of adults and regulated by the sex hierarchy. Using arrays that assay approximately one-third (4040) of *Drosophila* genes, we analyzed adults mutant for the regulatory genes *transformer* (*tra*), *dsx* and *fru* (Fig. 1). To select a small number of such genes for further study, we chose a conservative approach. Stringent statistical analysis of these data, combined with data from wild-type adults and adults that lack germline tissue (Arbeitman et al., 2002), identified 63 genes that are sex-differentially expressed in the adult soma and regulated by the somatic sex hierarchy. Additional selection criteria, and validation by RNA blot analysis, defined a set of 11 genes for further characterization. In situ hybridization revealed that sex-differential expression of all 11 genes is confined to the internal genitalia. Analysis of the regulation of these genes revealed that the sex hierarchy functions during development to specify their adult expression patterns, and that *dsx* probably functions in diverse ways to set their activities.

Materials and methods

Drosophila stocks

Flies were grown using standard conditions at 25°C, unless otherwise indicated. The wild-type stock was Canton S. XX *tra*, *XX Dsx^D* pseudomales, *fru* males and *dsx* intersexual mutant animals were *w^a/w; tra^1/Df(3L)st-j7*, *w/+;Dsx^D/dsx^{m+r15}* (XX), *fru$^{4-40}$/fru^{p14}* (XY), *w/+; dsx^{m+r15}/dsx^{d+r3}* (XX), and *w;dsx^{m+r15}/dsx^{d+r3}* (XY), respectively. *tudor* mutants are the progeny of virgin *tud^1 bw sp* females crossed to Canton S males. *tra2* temperature-shift experiments used the following genotypes: *B^{s}Y;tra-2^{ts1}/tra-2^{ts2}*(XY) and *tra-2^{ts1}/tra-2^{ts2}* (XX).

Microarray experiments

Microarray production, data acquisition and analysis were performed as described in (Arbeitman et al., 2002). Each hybridization was a comparison of one RNA sample to a reference sample that was comprised of RNA derived from whole animals representing all stages of the life-cycle. Normalization was calculated so that the average ratio of signals from the experimental and reference sample equalled one. Analyses were performed on log-transformed ratio values. In all cases, chromosomal males and females were sampled separately. The wild-type data set and *tudor* data set were described previously (Arbeitman et al., 2002); each time point was sampled in duplicate. The wild-type data set includes 0- to 24-hour-, 3-day-, 5-day-, 10-day-, 15-day-, 20-day-, 25-day- and 30-day-old adults. The *tudor* data set includes 0- to 24-hour- and 5-day-old adults. Microarray experiments on sex determination mutants *tra^1/Df(3L)st-j7* (XX), *fru$^{4-40}$/fru^{p1}* (XY) and *Dsx^D/dsx^{m+r15}* (XX) were performed with four (*tra* and *fru*) or five (*Dsx^D*) replicates. Microarray experiments performed on *dsx* null mutants (*dsx^{m+r15}/dsx^{d+r3}*) were sampled in duplicate.

Forced-choice statistical model

The expression levels of a gene in four experimental conditions were compared to determine whether they were more consistent with control by *dsx* or by *fru*. Each log-transformed data value for a gene was set as x_{ij}, where i=1,2,3 or 4 (for wild-type males, *fru* males, *tud* females and XX *dsx^D* pseudomales, respectively) and j=1,..., n_i replicates for the given genotype. If a gene is controlled by *dsx*, its expression level is expected not to differ between wild-type males and *fru* males; if it is controlled by *fru*, its expression level is expected not to differ between *tud* females and *dsx^D* pseudomales.

First, the within-group mean square (MS) was calculated assuming the gene was under *dsx* control. Three means were calculated:

$$\bar{x}_{tudF} = \frac{1}{n_3}\sum_{j=1}^{n_3} x_{3j}$$

$$\bar{x}_{dsxD} = \frac{1}{n_4}\sum_{j=1}^{n_4} x_{4j}$$

$$\bar{x}_{M} = \frac{1}{(n_1+n_2)}\left(\sum_{j=1}^{n_1} x_{1j} + \sum_{j=1}^{n_2} x_{2j}\right)$$

Then the sum of squared deviations of each data point from its respective mean was calculated and divided by the degrees of freedom:

$$MS_{DSX} = \frac{\sum_{j=1}^{n_3}(x_{3j}-\bar{x}_{tudF})^2 + \sum_{j=1}^{n_4}(x_{4j}-\bar{x}_{dsxD})^2 + \sum_{j=1}^{n_1}(x_{1j}-\bar{x}_{M})^2 + \sum_{j=1}^{n_2}(x_{2j}-\bar{x}_{M})^2}{(n_3-1)+(n_4-1)+(n_1+n_2-1)}$$

The MS, assuming *fru* control, was calculated in the same way, except that genotypes were expected to have the same expression level:

$$\bar{x}_{wtM} = \frac{1}{n_1}\sum_{j=1}^{n_1} x_{1j}$$

$$\bar{x}_{fruM} = \frac{1}{n_2}\sum_{j=1}^{n_2} x_{2j}$$

$$\bar{x}_{F} = \frac{1}{(n_3+n_4)}\left(\sum_{j=1}^{n_3} x_{3j} + \sum_{j=1}^{n_4} x_{4j}\right)$$

$$MS_{FRU} = \frac{\sum_{j=1}^{n_1}(x_{1j}-\bar{x}_{wtM})^2 + \sum_{j=1}^{n_2}(x_{2j}-\bar{x}_{fruM})^2 + \sum_{j=1}^{n_3}(x_{3j}-\bar{x}_{F})^2 + \sum_{j=1}^{n_4}(x_{4j}-\bar{x}_{F})^2}{(n_1-1)+(n_2-1)+(n_3+n_4-1)}$$

The MSs were then compared using an F test with the appropriate degrees of freedom.

RNA blot analyses

Total RNA was isolated with Trizol (Invitrogen), followed by RNeasy (Qiagen) or poly(A)+ isolation using Poly-ATtract (Promega). Blots were prepared from a Northern Max kit (Ambion). Radiolabeled RNA probes made with Strip-EZ kit (Ambion) were used at approximately 1-7×10^6 cpm/ml of hybridization solution. Blots were typically hybridized overnight at 68°C. Bound probes were visualized by phosphorimager (Molecular Dynamics).

Frozen section in situ hybridization

Frozen section in situ analysis was performed as described by Goodwin et al. (Goodwin et al., 2000).

Whole-mount in situ hybridization

Whole-mount in situ analysis was performed as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989), but with more extensive washes. Probes were made using the DIG RNA kit (Roche) and hydrolyzed to ~200 base pair fragments. Anti-digoxigenin FAB fragments conjugated to alkaline phosphatase (Roche), diluted 1:2000, were used.

Results and Discussion

Our goal was to identify genes sex-differentially expressed in adult somatic tissues and to explore their regulation by the sex hierarchy. This required both identifying genes expressed

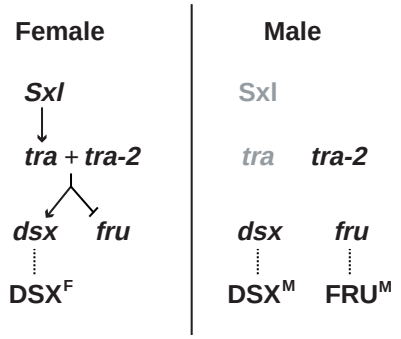


Fig. 1. Somatic portion of the sex determination hierarchy. In females, *Sxl* is activated and regulates the splicing of *tra* pre-mRNA, resulting in the production of TRA. TRA together with TRA2 regulates the female-specific splicing of *dsx* and *fru* pre-mRNAs. In males, *Sxl* is not activated and no functional TRA is produced, resulting in default splicing of *dsx* and *fru* pre-mRNAs. *dsx* produces both female- and male-specific isoforms. *fru* produces a male-specific isoform.

differentially in the two sexes, and determining whether that differential expression was in somatic tissues or in the germline. To this end, we performed a large-scale microarray screen, on approximately one-third of *Drosophila* genes, to examine relative gene expression levels in adult males and females that: (1) were wild type; (2) lacked a germline; or (3) were mutant for sex-determination genes. Microarray data on gene expression in wild-type males and females from eight different time points, and males and females that lack, or have greatly diminished, germline tissue (adult progeny of *tudor* mutants, referred to here as *tud* animals) (0- to 24-hour and 5-day), were taken from our previous study (Arbeitman et al., 2002). Expression data from 0- to 24-hour-old adults mutant for *tra*, *dsx* or *fru* were collected here using a two-color DNA microarray approach, as detailed previously (Arbeitman et al., 2002). These two data sets were comparable as the same microarray platform and common reference standard were used.

We identified genes that display sex-differential expression using data from seven of the eight wild-type adult time points. To maintain the independence of subsequent statistical tests, data from one time point, 0- to 24-hours, were reserved for subsequent comparisons. Here, 1,576 out of 4,040 genes showed significant sex-differential expression: 897 and 679 with higher expression in females and males, respectively ($P<0.001$ in a two-way ANOVA with sex and developmental time as main effects). We next used three separate one-tailed Student's *t*-tests to determine which of these 1,576 genes were likely to be sex-differentially expressed in the soma due to regulation by *tra* (see Fig. 1). All *t*-tests were carried out in duplicate, using both standard deviation and a global standard deviation (Jiang et al., 2001); genes had to pass both tests. The combination of the three tests was expected to yield a low false-positive rate.

The first and second *t*-tests asked which of the 1,576 genes were sex-differentially expressed in somatic tissue and downstream of *tra*, by comparing data from *tud* male and female animals ($P<0.05$), and from wild-type female and XX; *tra/tra* (hereafter called XX *tra*) individuals ($P<0.05$), respectively. We found 147 genes to be sex-differentially expressed in the soma and regulated by *tra*: 91 had higher expression levels in females and 56 had higher expression levels in males. We (Arbeitman et al., 2002) previously employed a test similar to the first *t*-test, but the final set of genes was limited to those with a greater than 2-fold expression difference, a criterion not applied here. The third test here required that, when a gene was expressed in both the soma and the germline, the germline expression was minor. We compared expression in wild-type females or males with that in same-sex *tud* animals, to identify genes that did not display significant differences in expression ($P>0.2$). Although this test re-uses data, it is purely conservative because it can only exclude genes from consideration. Of the 147 genes above, 73 genes passed this test, 37 and 36 with higher expression in females and males, respectively.

We confirmed the identity of the 73 genes by sequencing both ends of the cDNAs used to produce the microarray elements. We found ten cDNAs to be chimeric and removed these from further consideration, which left 63 genes that appeared to be expressed sex-differentially in somatic tissues and regulated by *tra*: 29 with higher expression in females and 34 with higher expression in males. Hereafter, we refer to these genes as female genes or male genes, respectively.

To compare the developmental expression profiles of these 63 genes, we generated a hierarchical cluster, which groups genes on the basis of similarities in their expression profiles (Fig. 2). The cluster included expression data from wild-type animals throughout development, and adult data from 0- to 24-hour and 5-day *tud* adults, 0- to 24-hour XX *tra* and XX dsx^{D} pseudomales, *dsx* null intersexual animals (XX and XY) and *fru* males that lack transcripts encoding FRU^{M}. Examination of the cluster showed that transcripts from about half of the female genes were present during the earliest stages of embryogenesis, suggesting that they are maternally contributed to the embryo, whereas the other half had their onset of expression during later zygotic stages. Many of the male genes were expressed before the adult stage, but their highest levels of expression were during adult life.

Validation

To confirm sex-differential somatic expression of the genes identified above, random samples of 10 genes from each of the male and female sets were tested on RNA blots of wild-type and *tud* male and female adults. The RNA blots confirmed the sex-differential somatic expression of seven male genes and four female genes (Tables 1 and 2; Fig. 3). For four of the seven confirmed male genes there was no evidence of expression in females, for the remaining three, there was a low level of expression in *tud* females, indicating expression in the female soma. For all four of the confirmed female genes there was evidence of low-level expression in the male soma as well. Of the remaining genes, two male and five female genes showed the expected sex-differential expression in wild-type tissue, but had equivalent expression in female and male *tud* tissue, suggesting that sex-differential expression in the germline, but not in the soma, may account for the differential expression observed in the microarray data (Fig. 3C). Expression of the tenth male gene did not agree with the microarray data; cDNA from this gene – used both for microarray preparation and as the RNA blot probe template – was subsequently shown to be

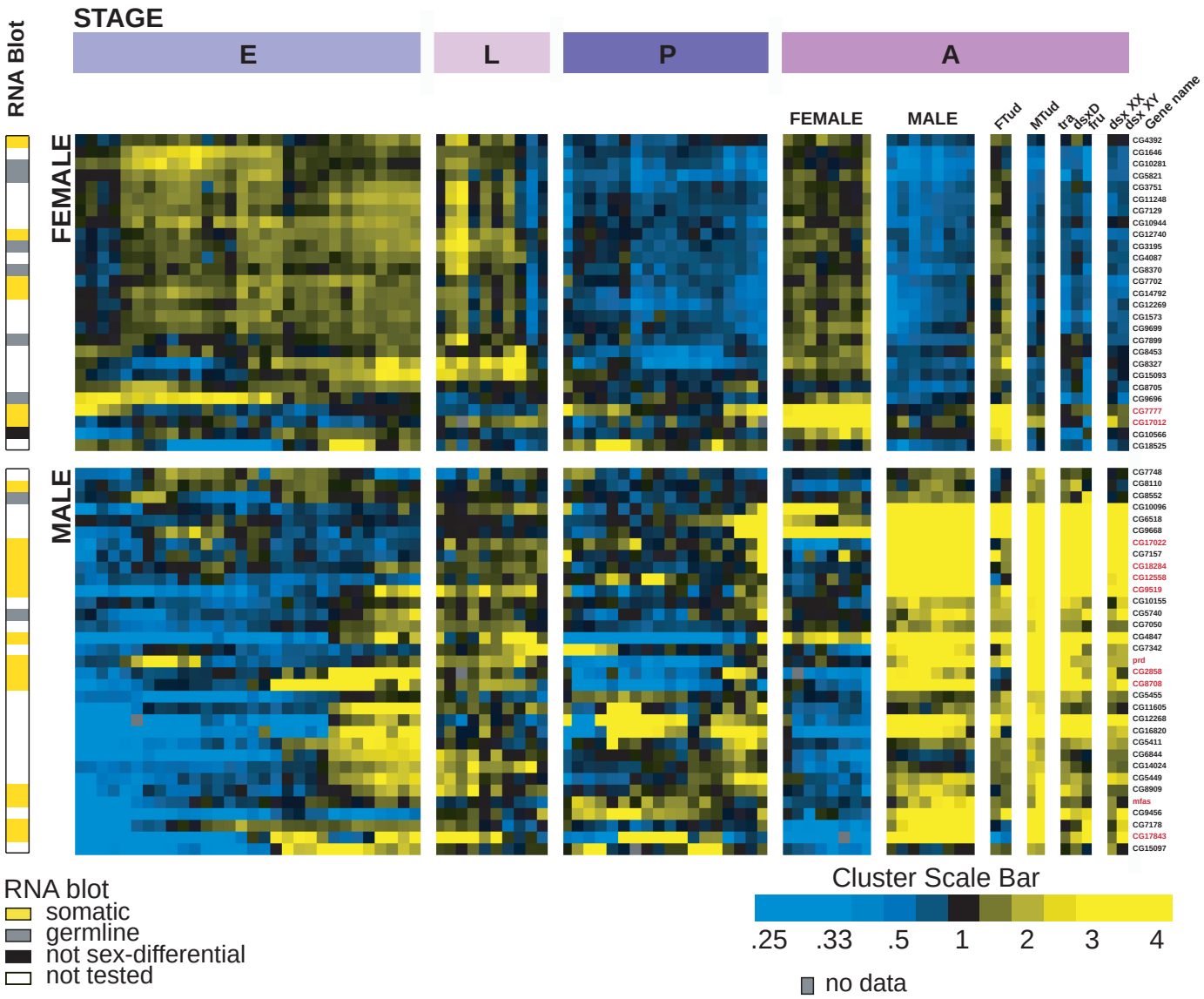


Fig. 2. Hierarchical cluster of female and male somatic genes. Genes were identified by statistical analysis, clustered hierarchically using Cluster and visualized with Treeview (Eisen et al., 1998). Compromised ESTs (chimeric or from a contaminated source) were removed for this analysis (see Table 2). The cluster includes whole animal data from wild-type animals at all stages of *Drosophila* development, including embryos (E), larva (L), pupae (P) and adults (A) (Arbeitman et al., 2002), and from the following mutants: *tud* female (FTud) and male (MTud); XX *tra* (tra); XX Dsx^{D} pseudomales (dsxD); *fru*; and *dsx* intersexual XY (dsxXY) and XX (dsxXX). Expression relative to the reference RNA is shown; yellow, blue and black indicate high, low and median levels of expression, respectively (see scale bar). Results of RNA blot analyses are shown (RNA blot), with key below. Gene names are shown on the right; red indicates genes chosen for further analysis.

Table 1. Summary of RNA blot analyses

Sex*	Selection for RNA blot analysis	Total ESTs assayed per category	Results		
			Somatic sex-specific	Germline sex-specific	Sex-nonspecific or inconclusive
Female	Random	10	4	5	1[†]
	Chosen	5	2	1	2[‡]
Male	Random	10	7	2	1[§]
	Chosen	7	7	0	0

*Female or male genes as identified by statistical analysis of microarray data.
[†]No apparent open reading frame.
[‡]One EST was sex-nonspecific and one from a contaminated well.
[§]EST was from a contaminated well.

Table 2. Summary of data for female and male genes

RAID***	EST†	CG‡	Blot§	Gene name¶	GO function**	FvM wt††	FvM tud††	Model‡‡	Model P§§	FvdsxD¶¶	MvFru¶¶
Female genes											
75H3	–		–			85.32	107.16	DSX	*	*	
79G7		CG17012	Yes		Peptidase	17.91	18.49	DSX	*	*	
64B10	–		–			17.91	32.10	DSX	*	*	
61G12	–		No			6.67	13.97	DSX	*	*	
75H7	–		No			4.43	11.49	DSX	*	*	
86D9	LD27313	CG7777	Yes		Transporter	3.64	3.15	DSX	*	*	
75G4	GH23040	CG1090	–		Transporter	3.60	5.76	DSX	*	*	
88A8	–		–	sop	Ribosomal_protein	3.21	2.21	DSX		*	
88A7		CG5821	G		RNA_binding	2.91	2.25	DSX		*	
60F4	GH08387	CG8327	–		Enzyme	2.55	3.51	DSX		*	
85C11	LD26157	CG10281	G	TfIIFalpha	Transcription_factor	2.45	2.01	DSX		*	
98D8		CG14792	Yes	sta	Ribosomal_ protein	2.43	1.81	FRU		*	*
45G12	–		–			2.41	1.40	DSX		*	
109G12		CG1573	–	Pgk	Protein_kinase	2.35	2.09	DSX		*	
85E11	LD26426	CG1646	–		DNA_binding	2.31	1.67	DSX		*	
98H3		CG10944	–	RpS6	Ribosomal_protein	2.22	1.87	FRU		*	
89B6		CG12269	–		Unknown	2.22	1.77	FRU			*
107B5	LD47943	CG7899	G		Enzyme	2.14	1.91	DSX			
74E3	–		–			2.10	2.25	FRU			
107A9	LD47858	CG8370	G		Unknown	2.06	2.10	DSX			
72B11		CG3195	G	RpL12	Ribosomal_protein	2.06	2.03	DSX			
109F12	LP06017	CG9699	–		Cytoskeletal_structural_protein	2.06	1.75	FRU		*	
40E11	–		–			2.06	2.10	DSX			
49G4	LD35056	CG9696	G		DNA_binding	2.04	1.56	DSX		*	
102H9	LD44094	CG11248	–		Motor_protein	2.04	1.97	DSX			
78D9		CG3751	–		Ribosomal_protein	2.03	1.96	FRU			
102H11	LD44138	CG7129	–		Signal_transduction	1.95	1.54	DSX			
75H6	GH23156	CG10566	No		Defense/immunity_protein	1.89	4.93	DSX	*	*	
109H12	LP06937	CG7702	Yes		Cell_adhesion	1.86	1.57	FRU			*
65D7	GH13422	CG4087	–	RpP2	Ribosomal_protein	1.85	2.16	DSX			
83B2	GM13756	CG12740	Yes		Ribosomal_protein	1.80	1.82	DSX			
78D7	GH25190	CG4392	Yes		Transporter	1.79	1.66	FRU			
41E4	GH14439	CG18525	–		Unknown	1.79	2.83	DSX	*		
41H5	GH14851	CG8453	–	Cyp6g1	Cytochrome_P450	1.67	1.50	DSX			
68B6	–		–			1.66	1.91	DSX			
34G1	GH06693	CG9547	–		Acyl-CoA_dehydrogenase	1.58	1.86	DSX			
96E9	LD37170	CG8705	–		Cytoskeletal_structural_protein	1.38	1.44	DSX			
Male genes											
38G8	GH11288	CG7157	Yes	Acp36DE	Signal_transduction	218.37	88.83	DSX	*	*	*
65F5	GH13755	CG17022	Yes		Unknown	143.95	76.82	DSX	*	*	
38A9	GH10507	CG18284	Yes		Unknown	62.70	68.39	DSX	*	*	
75E2	GH22889	CG17843	Yes		Electron_transfer	36.55	15.64	DSX	*	*	
54C10	GH01346	CG10096	–		Unknown	15.51	4.25	DSX		*	
70E7		CG8708	Yes		Unknown	11.72	16.46	DSX	*	*	
68B5	GH15905	CG12558	Yes		Endopeptidase	11.62	11.39	DSX		*	
77H12		CG7178	Yes			9.78	6.66	FRU		*	
76E9		CG9519	Yes		Enzyme	9.40	7.38	DSX	*	*	
68B9	GH15921	CG16820	–		Unknown	8.22	4.39	DSX		*	
96C7	–		Yes			6.72	12.60	DSX	*	*	
54F6	–		No			6.66	5.00	DSX		*	
30E3	–		Yes		Endopeptidase	6.13	2.04	FRU		*	
42C7	GH15295		–			6.10	9.91	DSX		*	*
65E3	GH13557	CG7342	–		Transporter	4.92	4.04	DSX		*	*
77F5	GH24480	CG12268	–		Enzyme	4.64	2.17	DSX		*	
32E4	GH04125	CG9456	–		Serpin	4.30	2.15	DSX		*	
77H9	GH24781	CG6518	–	inaC	Protein_kinase	4.23	1.30	DSX		*	
66D12	GH14506	CG8909	Yes		Receptor	3.16	2.17	DSX	*	*	
75B4	GH22686	CG6716	Yes	paired	Transcription_factor	3.07	2.31	DSX		*	
74C9	GH21941	CG5449	–		Receptor	2.83	2.14	DSX		*	
34H9		CG3359	Yes	mfas	Signal_transduction	2.78	1.79	FRU		*	
40A5			–	Rh4	G_protein_linked_receptor	2.69	1.55	DSX		*	
54A10	–		–		Signal_transduction	2.33	1.45	DSX		*	
72H5	–		–			2.24	1.31	FRU		*	
72B3	GH19969	CG8110	Yes		Signal_transduction	2.12	2.10	DSX			
102D1	LD43488	CG5740	G		Motor_protein	2.02	1.40	FRU			
39D8	GH11985	CG14024	–		Unknown	2.01	1.58	DSX		*	
73F7	GH21295	CG5411	–		Enzyme	1.97	1.31	FRU		*	
55F5	GH03013	CG11605	–		Actin_binding	1.95	1.37	DSX		*	
70E1	GH18278	CG15097	–		Actin_binding	1.89	1.42	DSX		*	
61G11	GH09582	CG6844	–	nAcRa-96Ab	Ion_channel	1.78	1.49	DSX			
67D4	GH15286	CG5455	–		Transcription_factor	1.66	1.36	DSX		*	
81F10	GH27701	CG8552	G		Ligand_binding_or_carrier	1.59	1.41	FRU			
34A10	GH05937	CG7050	–		Cell_adhesion	1.57	1.64	DSX		*	
40F6	GH13452	CG7748	–	OstStt3	Enzyme	1.53	2.48	DSX		*	

***An arbitrary unique identifier (Arbeitman et al., 2002).

†The BDGP unique identifier, if known. All BDGP ESTs were re-sequenced and those that did not match the expected EST have no designation. A '–' indicates that the EST is chimeric or from a contaminated well. 75H3 is a CG6666/CG3244 chimera; 64B10 is a*gbb*/*Yp1* chimera; 61G12 has two sequence populations in well; 75H7 has no apparent open reading frame; 88A8 is chimeric with non-*Drosophila* DNA; 45G12 is a ribosomal/P-element chimera; 74E3 is a ribosomal/no significant BLAST chimera; 44E11 is aCG7283 (ribosomal)/CG8536 (B-N-acetylglucosaminyl-glycopeptide β-1-4 galactosytransferase); 68B6 is a ribosomal/ion channel chimera; 96C7 has two sequence populations in well; 30E3 is a CG5023/CG4651 chimera; 54A10 and 72H5 contain a transposon sequence; 98D8 contains sequence from ribosomal and CG9243; 40A5 is an EST from either RH4 or sina, which are coded for on opposite strands; 74E3 contains sequence from ribosomal and CG6684; 40E11 chimera of two ribosomal genes.

‡The celera-predicted gene based on the EST sequencing results presented here.

§Results of RNA blot analysis. 'Yes', 'No' and 'G' indicate that the RNA blot confirms or does not confirm the somatic sex differential expression, or is due to germline expression, respectively.

¶The names of genes for those genes that have been previously studied.

**Predicted protein functions of the products of the ESTs.

††FvMwt and FvMtud, the fold difference in expression between all of the adult wild-type females and males, and all of the adult tudor female and male microarray data, respectively.

‡‡The branch of the hierarchy that is predicted to regulate the gene, based on the statistical model presented (see Materials and methods).

§§An asterisk indicates that the *P* value is less that 0.05 based on the model.

¶¶An asterisk indicates that in one-tailed *t*-tests the *P* value is less than 0.05. The data in the tables are sorted: those showing the largest fold difference in expression between adult wild-type females and males are at the top, and the rest of the data is in descending order based on that criterion.

from a contaminated source and was removed from the study set. Finally, RNA blot analysis of the tenth female gene revealed no signal, even at very low hybridization stringency; a subsequent analysis of its sequence indicated there was no open reading frame for this predicted gene. Extrapolation of these findings predicts that about 60% (11/18) of the 63 genes identified in the microarray analysis will be confirmed as targets of the somatic sex hierarchy. Following the validation of randomly selected genes, we chose additional genes from the set of 63, using the criteria described below, and examined these by RNA blot analysis as well (Table 1). In all, 32 of 63 genes were so examined and RNA blot analyses have confirmed that 20 of these are expressed sex-differentially in somatic tissue.

Comparison of gene sets from two studies

The set of genes defined here partially overlaps the set of 68 genes we previously defined as sex-differentially expressed in somatic tissues (Arbeitman et al., 2002), resulting in three categories of genes: (1) those exclusively identified in the previous study (33 genes); (2) those identified in both studies (29 genes); and (3) those exclusively identified in this study (34 genes). We investigated whether differences between the gene sets in the two studies were due only to differences in the sample size and statistical approaches employed, or whether there may also have been a biological basis, given the addition of *tra* mutant data in our current study. For the genes identified in just one study, we examined the data and found that nearly all genes were excluded from the other study for non-biological reasons. Of the 33 genes identified only in the previous study, five genes were excluded from the current study because microarray values were not available, and 24 genes were excluded because a single *P*-value fell just above the statistical threshold of this study. Of the 34 genes identified exclusively in the current study, 29 had a <2-fold difference in expression between male and female *tud* flies, and were therefore excluded by the previous study; the remaining five genes were excluded from the previous study because it used a slightly more stringent *P*-value cutoff in the male versus female *tud t*-test.

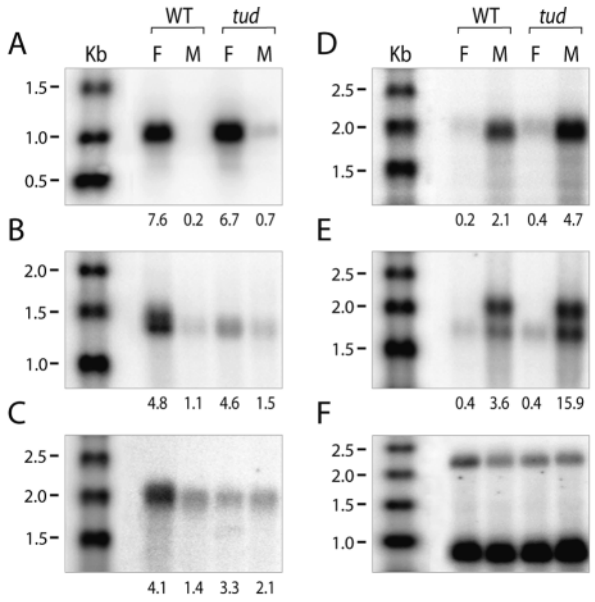


Fig. 3. (A-E) Characteristic examples of RNA blot analyses. Each blot consists of 3 μg polyA$^+$ RNA per lane from female (F) or male (M) 5-day-old adult animals: wild type (WT) and *tud*. Microarray values for each gene (see Materials and methods) are included below the respective lanes. Probes were derived from ESTs for female genes (A-C) or male genes (D-E). (A) CG17012, (D) CG2858 and (E) CG8708 agree with microarray data. (B) CG7777 agrees with microarray data in that there is somatic expression (slight elevation in expression of female over male *tud* lanes). Germline expression is also apparent (much greater intensity of wild-type female over *tud* female bands). (C) CG7899 expression is sex-specific, but is due to germline rather than somatic expression. (F) Internal controls. Two genes, each with similar levels of female and male expression according to the microarray data, were used as internal control probes: CG4659 (Srp54K) (upper band) and CG10664 (cytochrome c oxidase subunit IV). Expression levels in the wild-type female lane of CG4659 and the *tud* male lane of CG10664 are typically slightly elevated.

Microarray data for four genes identified exclusively in the previous study indicate that those four genes may be sex-differentially expressed in somatic tissues, but not regulated by *tra*. On sequencing the ends of these genes to confirm their identities, two were found to be from contaminated sources and were not considered further. One remaining gene, more highly expressed in males, was found to be *RNA on the X 1* (*roX1*), which is involved in the male-specific process of dosage compensation and is known not to be regulated by *tra* (reviewed by Meller and Kuroda, 2002). The other remaining gene, more highly expressed in females, was CG9709, which encodes Acox57D-d (an enzyme expressed in the peroxisome). CG9709 was not further validated by RNA blot analysis and we do not have the statistical power to say with high confidence that it is not controlled by *tra*.

Selecting genes for further study

We next chose a subset of 11 of the 63 genes for in-depth analysis, based on whether the gene: (1) showed a large difference in expression between the sexes; (2) had biology of interest to us; and (3) showed strong evidence of regulation by *dsx* or *fru*. All 11 genes met the first criterion, showing expression in one sex with little or no expression in the other sex by RNA blot (Fig. 3 and Table 2). Only two showed a <3-fold difference between *tud* females and males by microarray analysis (see Table 2): *midline fasciclin (mfas)*, which encodes a signal transduction protein, and *paired (prd)*, which encodes a transcription factor. The published information on these genes, and their likely regulatory roles, favored their inclusion. Other genes considered biologically interesting were those that suggested ties to existing knowledge of *Drosophila* reproductive biology, or that were homologs of mammalian genes involved in disease processes (see next section).

To identify genes by the third criterion we used two approaches. First, *t*-tests were used to compare microarray data between wild-type females and XX *dsx*^{D} pseudomales, or wild-type and *fru* males. Second, because of low sample sizes, and to avoid identifying genes based on strain-specific differences, a statistical model was developed that predicted either *dsx* or *fru* regulation for each gene (see Materials and methods and Table 2). As both approaches reanalyze data used previously, the results are heuristic. Of the 11 genes ultimately selected, 10 were significant for regulation by *dsx* according to one or both approaches. The exception was the male gene *mfas*, which

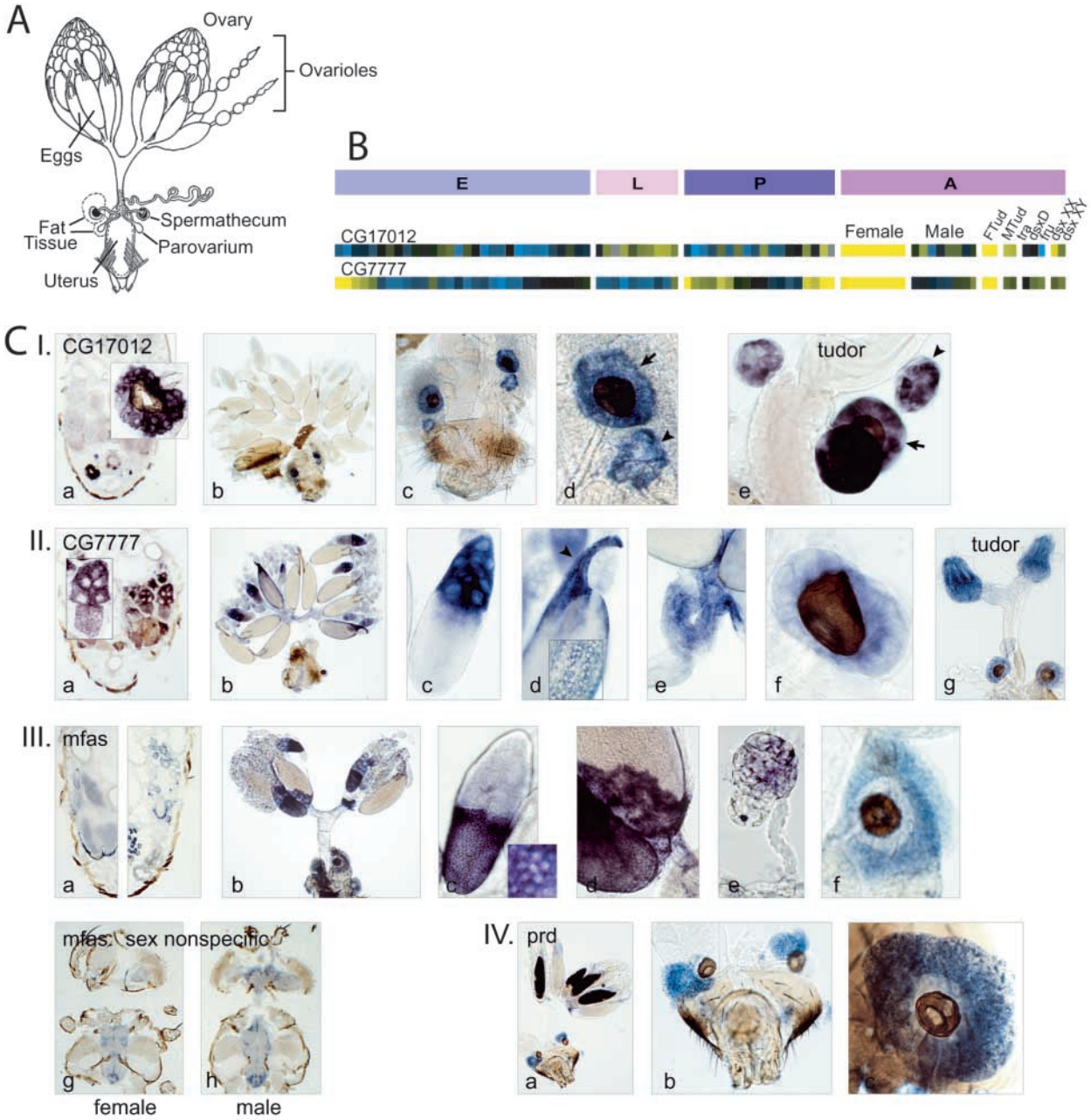


Fig. 4. Temporal and tissue-specific expression in female internal genitalia. (A) Schematic of the internal female reproductive system. Modified, with permission, from Miller (Miller, 1950). (B) Expression profiles of two female genes (see Fig. 2 legend). (C, parts I-IV) In situ hybridization of wild-type and *tud* 5-day adult female reproductive tissues using probes derived from female (I,II) and male (III,IV) genes. I part a, II part a, and III parts a, h and i, are frozen tissue frontal sections of whole animals; all other images are from whole-mount in situ hybridization analysis. (I) Expression is seen in spermathecae (arrows) and parovaria (arrowheads) in wild-type (a-d) and *tud* (e) tissues. (II) Expression in nurse cells of stage 8-10 egg chambers (a-c); in follicle cells covering an early stage 14 egg chamber, including the dorsal appendage (arrowhead; inset is at a closer focal plane; d); in oviducts (e); in spermathecae of wild-type (f) and *tud* (g) tissues; and in sheath of the immature ovaries found in *tud* animals (g). (III) Expression of male-enriched *mfas* in female tissues (a-f): in follicle cells of stage 9-10 egg chambers (b,c); in sloughed off follicle cells of a later stage egg chamber (d); in parovaria (e); in fat tissue (f); and, sex-nonspecifically, in CNS (g,h). (IV) Expression of male-enriched *prd* in female fat tissue (a-c). Insets are digital magnifications unless otherwise noted.

the forced-choice model found (though not with significance) more likely to be regulated by *fru*, and which is known to function in the central nervous system (Hu et al., 1998), as do the male-specific *fru* products. Having chosen a set of 11 genes, two female and nine male, we proceeded to determine their patterns of expression by in situ hybridization.

Tissue-specific adult expression

We determined the expression patterns of the 11 genes by in situ hybridization to frozen sections and to wholemounts of dissected tissue from 5-day-old wild-type adults. In all cases, the direction of sex-differential expression agreed with both microarray and RNA blot analyses, and was due to expression

in the sex-specific tissues of the internal genitalia. Of the 11 genes, nine were expressed exclusively in one sex or the other, and two were expressed in both sexes with overall expression higher in males. Two genes, in addition to sex-differential expression in the internal genitalia, were expressed sex-nonspecifically in tissues outside the genitalia.

Female genes

The internal reproductive system of females consists of ovaries, oviducts, three sperm storage organs (the sperm receptacle and the paired spermathecae), paired parovaria (also known as female accessory glands), the uterus and vagina (Miller, 1950) (Fig. 4A). Spermathecae store sperm, but have not been well studied at the molecular level. Among the insects in which parovaria have been studied, functions of their secretions vary, and, in other Diptera, include antibacterial action (Marchini et al., 1991) and increased sperm penetration of the egg (Leopold and Degrugillier, 1973). Spermathecae and parovaria are somatically derived, surrounded by fat body and connected by short ducts to the uterus.

Strong expression of CG17012 was detected in both the spermathecae and parovaria (Fig. 4C, part I). In some individuals, weak expression was also found in the fat body surrounding the spermathecae (not shown). Transcripts were observed in the single-cell epithelial layer that surrounds the mushroom-shaped cuticle of the spermathecae, and in the single-cell wall surrounding the lumen of the parovaria. Germline presence was not required for this expression; signal was also seen in *tud* animals (Fig. 4C, part I, part e). CG17012 contains a conserved trypsin-like serine protease domain (Blastp, expect 7e-47, 230 residue domain, 100% aligned). CG17012 could induce sperm motility, as trypsins do in Lepidoptera (Friedlander et al., 2001). A third alternative is that in *Drosophila*, proteolytic cleavage of a male-produced putative pro-hormone occurs in the mated female (Park and Wolfner, 1995), and CG17012 could be involved in this process. Alternatively, CG17012 protein might offset harmful effects of male seminal proteins, some of which reduce female viability (reviewed by Wolfner, 2002). Interestingly, Acp62F, a male-produced trypsin inhibitor found in the sperm storage organs of mated females, increases mortality when ectopically expressed in females (Lung et al., 2002).

CG7777 was expressed in both somatic and germline tissues (Fig. 4C, part II), as predicted by RNA blot analysis (Fig. 3B). CG7777 mRNA was detected in spermathecae and oviducts (Fig. 4C, part II, parts e,f), and in egg chambers at most stages. In stages 8 through 10, expression was strong in nurse cells (germline origin) (Fig. 4C, part II, part c). By stages 13 and 14, when the chorion is being secreted by follicle cells (somatic origin), expression was seen in follicle cells – most strongly in those of the dorsal appendage (Fig. 4C, part II, part d). In *tud* animals, CG7777 message was apparent in spermathecae, and in the peritoneal or epithelial sheath of immature ovaries or ovarioles, respectively (Fig. 4C, part II, part g). CG7777 is predicted to encode an aquaporin, a member of the MIP (Major Intrinsic Protein) family of transmembrane transporters (reviewed by Verkman, 2002), and is most similar to aquaporins 1 and 4 (Blastp, e value 1e-35) in humans.

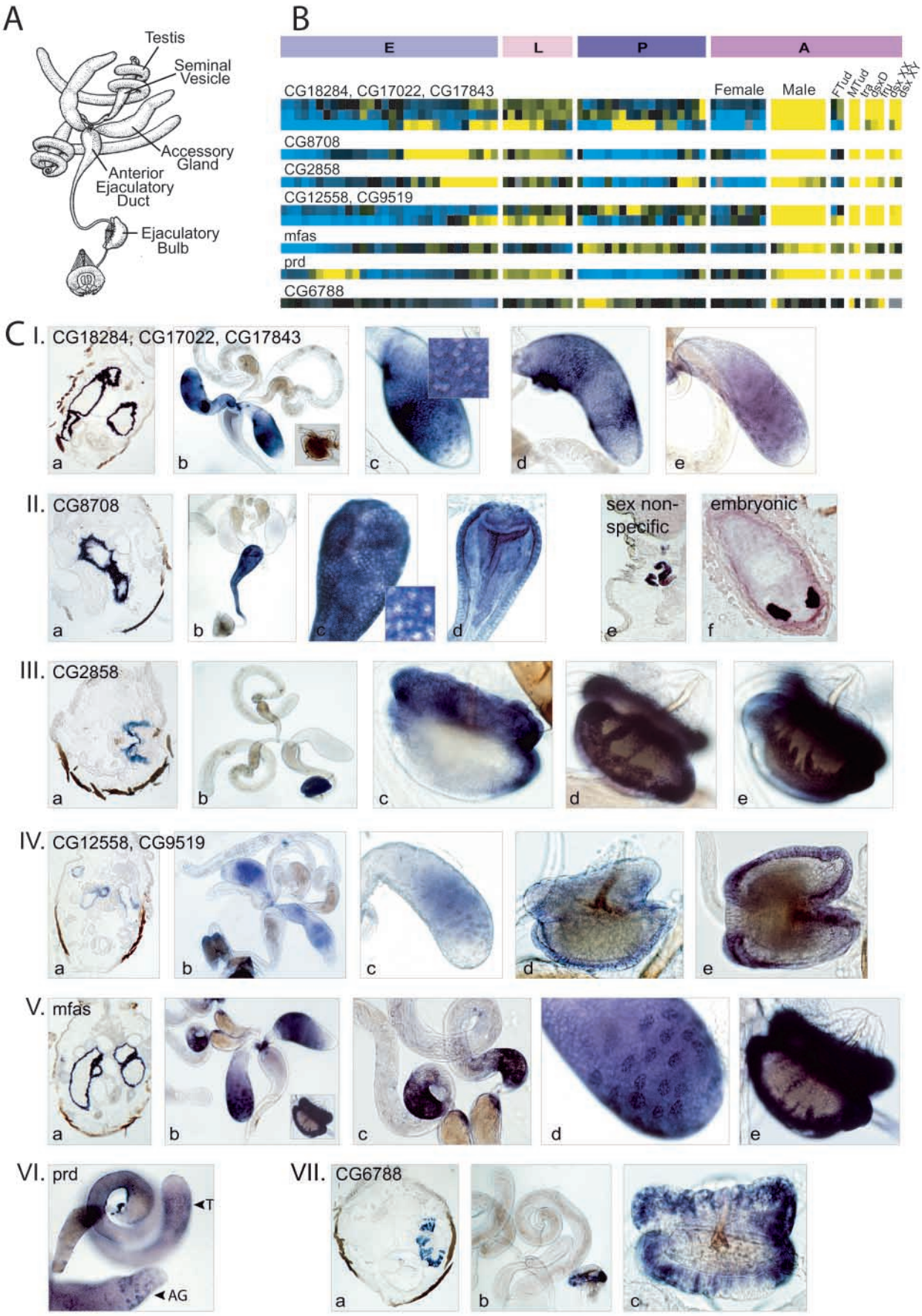
Male genes

The internal male reproductive system includes the testes, seminal vesicles, accessory glands, ejaculatory duct and ejaculatory bulb (or sperm pump) (Bairati, 1968) (Fig. 5A). Secretions from the male accessory glands perform many functions in the mated female, including decreasing her receptivity to mating, increasing ovulation and egg production, and enhancing sperm survival and storage (reviewed by Wolfner, 2002). Sperm, accessory gland secretions and ejaculatory duct secretions are propelled forward from the ejaculatory duct to the ejaculatory bulb, which adds to the propulsion of seminal fluid and contributes secretions of its own (Bairati, 1968). Five genes were expressed exclusively in one organ and we describe these first.

Expression of three genes (CG18284, CG17022 and CG17843) was detected only in the male accessory glands. Each gland consists of a lumen bordered by a single layer of secretory cells, which is in turn surrounded by a muscle sheath. The majority of the secretory cell layer is comprised of hexagonal and binucleate ‘main cells’. Interspersed among these, toward the distal end of each gland, are large, round, binucleate ‘secondary cells’ (Bairati, 1968; Bertram et al., 1992). The two cell types secrete partially overlapping sets of products.

CG18284 was strongly expressed in the main cells and at the junction of the accessory glands to the ejaculatory duct (Fig. 5C, part I, parts a-c). CG18284 is similar to several lipases, including, in *Drosophila*, *Lip1* and *Lip3* (Blastp, e value 3e-63 and 2e-62, respectively). *Lip1* expression is not sex-differential in adults and may be a digestive enzyme, whereas *Lip3* is not expressed in the adult (Pistillo et al., 1998). Previous studies have reported strong triacylglycerol lipase activity in accessory glands, and transfer of this activity to mated females (Smith et al., 1994). Six lipases in the *D. melanogaster* genome have been identified through homology searches using *D. simulans* accessory gland ESTs (Swanson et al., 2001). Two of these, CG17101 and CG17097, are quite similar to CG18284 (Blastp, e value 2.1e-190 and 1.1e-111, respectively), and all three map to the same chromosomal region (2L, ~31F5). Swanson et al. suggest that lipases contributed by the male to the mated female might be involved in lipid nutrient metabolism or in fusion of spermatazoa and eggs (Swanson et al., 2001).

Fig. 5. Temporal and tissue-specific expression in male internal genitalia (A) Schematic of the internal male reproductive system. Modified, with permission, from Miller (Miller, 1950).
(B) Expression profiles of male genes (see Fig. 2 legend) (C) In situ hybridization of wild-type 5-day-old (I-VI), or 0-24 hour (VII), adult and mutant *tud* male reproductive tissues using probes derived from male genes. I-V part a, VII part a, and II parts e and f are frozen tissue frontal sections of whole animals; all other images are of whole-mount tissues. (I) Expression in the accessory glands: CG18284 (a-c), CG17022 (d) and CG17843 (e). (II) Expression of CG8708 in the anterior ejaculatory duct (a-d; c and d are different focal planes), including the papilla (d), and, sex-nonspecifically, in adult and embryonic salivary glands (e,f). (III) Expression of CG2858 in the ejaculatory bulb (a-e), showing individual differences in expression (c-e). (IV) Expression in accessory glands and the ejaculatory bulb: CG12558 (a-d) and CG9519 (e). (V) *mfas* expression in testes (a-c) and on the surface of seminal vesicles where the testes attach (c); in accessory glands (a,b,d); and in the ejaculatory bulb (b,e). (VI) *prd* expression in testes (T) and accessory glands (A). (VII) Expression of CG6788 in 0-24 hour ejaculatory bulb (a-c). Insets are digital magnifications.


A
Testis
Seminal Vesicle
Accessory Gland
Anterior Ejaculatory Duct
Ejaculatory Bulb
B
E
L
P
A
Female
Male
FTud
MTud
tra-D
dsx-D
tra-X XY
dsx XY
CG18284, CG17022, CG17843
CG8708
CG2858
CG12558, CG9519
mfas
prd
CG6788
C I. CG18284, CG17022, CG17843
a
b
c
d
e
II. CG8708
sex non-specific
embryonic
f
III. CG2858
IV. CG12558, CG9519
V. mfas
VI. prd
T
AG
VII. CG6788

CG17022 was strongly expressed in the accessory gland (Fig. 5C, part I, part d). Its predicted protein is serine-rich, with no significant similarities to known proteins.

CG17843 (Fig. 5C, part I, part e) is conserved in the two canonical domains of the recently proposed QSOX family of sulfhydryl oxidases and has been named dmQSOX2 (reviewed by Thorpe et al., 2002). In many organisms, QSOX are known to localize to intracellular and extracellular spaces of secretory tissues, where they are thought to catalyze and maintain the formation of disulfide bonds in secreted proteins. In addition, they may participate in forming the extracellular matrix of these tissues. Interestingly, sulfhydryl oxidase message has been found in the male rat genital tract, where proposed functions include protecting spermatozoa from microbial or sulfhydryl degradation (Benayoun et al., 2001). Accessory gland expression of CG17843 is consistent with these suggested functions.

CG8708 is expressed strongly and exclusively in the epithelium of the anterior ejaculatory duct (Fig. 5C, part II, parts a-d). CG8708 is similar to a class of β3-galactosyltransferases in a variety of organisms (Blastp, e value 1e-73 in *C. elegans* and mouse, for example). The putative human ortholog, which shows 41.3% similarity to CG8708, has been identified as core 1 UDP-galactose:N-acetylgalactosamine-alpha-R β1,3-galactosyltransferase (core 1 β3-Gal-T) (Ju et al., 2002a), which catalyzes the last step in the formation of the core 1 structure – a precursor for many membrane-bound and secreted mucin-type glycoproteins. In rat, the shorter of two transcripts for core 1 β3-Gal-T was previously found in the testis only; humans have two transcripts as well (Ju et al., 2002a; Ju et al., 2002b). We also found two transcript size classes of CG8708 on RNA blots (Fig. 3E). Both sizes were detected in the male soma and a trace amount of the shorter transcript was also found in the female soma. Frozen section in situ analysis detected sex-nonspecific expression of CG8708 in the salivary glands of adults (Fig. 5C, part II, part e), and of a stage 13 embryo found within an adult female (Fig. 5C, part II, part f), suggesting a developmental role.

In the ejaculatory bulb, CG2858 was highly expressed but in variable patterns between the horns of each lobe and in streaks of cells extending up the sides of each lobe (Fig. 5C, part III, parts c-e). CG2858 contains a male sterility (MS2) domain (Blastp, expect 3e-56, 196 residue domain, 100% aligned), first identified in *Arabidopsis thaliana* (Aarts et al., 1997) and now known in many organisms. In the jojoba seed, MS2 has been characterized as a fatty acyl-coA reductase that is involved in the biosynthesis of wax storage lipids (Metz et al., 2000). *Drosophila* ejaculatory bulb secretions contribute substantially to the formation of the waxy mating plug in the mated female (Bairati and Perotti, 1970; Lung and Wolfner, 2001). These secretions include Protein Ejaculatory Bulb-melanogaster (PEB-me; PEB – FlyBase), which may contribute to the mating plug structure (Lung and Wolfner, 2001), and the lipid cis-vaccenyl acetate (Brieger and Butterworth, 1970). The discovery of a putative MS2 protein suggests identification of an enzymatic contributor to the waxy mating plug structure.

Two genes, CG12558 and CG9519, were detected in both the accessory glands and ejaculatory bulb (Fig. 5C, part IV). CG12558 (Fig. 5C, part IV, parts a-d) encodes a predicted endopeptidase with a trypsin-like serine protease domain (Blastp, expect 4e-12, 88% aligned). To date, nine putative proteases have been identified among *Drosophila* male accessory gland proteins (reviewed by Wolfner, 2002). Lack of identity to these proteases suggests CG12558 is the tenth such protein.

CG9519 (Fig. 5C, part IV, part e) contains a conserved choline dehydrogenase domain (Blastp, expect 1e-94, 542 residue domain, 98.5% aligned). Although CG9519 is annotated as a choline dehydrogenase (The FlyBase Consortium, 2003) and is similar to human choline dehydrogenase (5e-76), it also shares a high degree of identity with *Drosophila* glucose dehydrogenase (GLD) (Blastp, e value e-102). GLD has previously been shown to be regulated by the sex hierarchy (Feng et al., 1991) and transferred to females during mating (Cavener and MacIntyre, 1983). However, the pattern of localization we found for CG9519 differs from that of GLD in *D. melanogaster*; GLD is present only in the ejaculatory duct of males and is also expressed in the female reproductive tract (Cox-Foster et al., 1990; Feng et al., 1991).

mfas encodes a protein containing fasciclin and β-Ig-H3 domains, which are thought to mediate cell adhesion (Hu et al., 1998). *mfas* transcript was detected in both sexes (although at higher levels in males than females) according to both microarray and RNA blot analyses, and was detected in the internal genitalia of both sexes. In males, *mfas* transcript is strongly expressed in a region of each testis extending approximately one tenth its length from the proximal end, and in a small area around the testis attachment site on the seminal vesicle (Fig. 5C, part V, part c), in both main and secondary cells of the accessory glands, at the junction with the anterior ejaculatory duct (Fig. 5C, part V, parts d and b, respectively), and in the ejaculatory bulb in a pattern similar to that described for CG2858 (Fig. 5C, part V, part e). In females, prominent signal was detected in follicle cells that surround oocytes of stage 9 and 10 egg chambers (Fig. 4C, part III, part c), until later stages when these cells are sloughed off (Fig. 4C, part III, part d). Weak signal was seen in parovaria of some individuals (Fig. 4C, part III, part e), and in fat tissue surrounding the spermathecae (Fig. 4C, part III, part f). The expression of *mfas* in a region of the gonad containing mature germline cells in both sexes, combined with its predicted role in cell adhesion, raises the possibility that it may function to mediate germline/somatic interactions prior to release of the sperm or egg into the seminal vesicle or oviduct, respectively. In addition, *mfas* was expressed in the central nervous system (CNS; Fig. 4C, part III, parts g,h); previous analyses demonstrated that *mfas* is expressed on the midline neurons and glia in the embryo (Hu et al., 1998). At the level of resolution of our analysis, we are unable to detect expression differences between males and females in the CNS.

Finally, in males we detected *prd* in the accessory glands and, at low levels, in the testes (Fig. 5C, part VI). In females, *prd* is strongly expressed in the fat body surrounding the spermathecae (Fig. 4C, part IV), although this expression appears variable among individuals. Localization of *prd* to male accessory glands had previously been documented (Bertuccioli et al., 1996), but expression in the testes and female fat body had not been reported. *prd* encodes a transcription factor in the Pax homeodomain gene family. In

addition to its role in activation of segment-polarity genes, *prd* is involved in the development of adult male accessory glands and in the regulation of at least three accessory gland proteins (Xue and Noll, 2002).

Identification of a gene, outside our derived data set, with a sex-specific developmental expression profile

Any set of criteria designed to identify genes from microarray studies may exclude some genes of legitimate interest. Here, for example, visual inspection of the adult microarray data (Fig. 5B), and subsequent in situ analysis, identified CG6788, expressed only during the 0- to 24-hour stage of adult male life. This gene was not identified above because the first ANOVA test used to identify genes did not use the 0-24 hour time point. CG6788 encodes a predicted cell adhesion protein that contains a fibrinogen domain (Blastp, expect 4e-59, 215 residues, 92.1% aligned) with similarity to angiopoietin-like 1 precursor (Blastp, expect 3e-37). This gene is expressed in the male ejaculatory bulb at the 0- to 24-hour stage (Fig. 5C, part VII), but not in 5-day-old males. Expression was observed in a striped pattern in the epithelial cells that border each lobe. The timing of CG6788 expression suggests that it might play a role in ejaculatory bulb development rather than a physiological role.

On the nature of sex-differential gene expression in adults

Previous microarray studies in *Drosophila* showed that about half the genome is sex-differentially deployed (Jin et al., 2001; Arbeitman et al., 2002; Parisi et al., 2003; Ranz et al., 2003). In *C. elegans*, a smaller percentage of the genome is so deployed (12%) (Jiang et al., 2001). Our results indicate that, in *Drosophila*, less than 1.5% of the genome is sex-differentially regulated by *tra* in the soma; however, because our statistical approach is conservative, this is likely to be an underestimate. In *C. elegans*, the fraction of the genome sex-differentially deployed in somatic tissue is also small [based on Jiang et al., the low estimate is 3-4% (Jiang et al., 2001)]. Thus in two very different animals it appears that sex-differential transcription in the adult is largely due to germline expression, with a much smaller set of genes sex-differentially expressed in the soma.

Our 63-gene set was defined as those genes whose somatic sex-differential expression is downstream of *tra*. We used a forced-choice statistical model to determine whether *dsx* or *fru* was more likely to regulate each gene in our subset and found that, for 58 genes in our 63-gene set, and for 10 of the 11 genes in the group chosen for further study, sex-differential expression is more likely to be a consequence of *dsx* activity than *fru* (Table 2). This indicates that the approach taken was very successful in identifying genes sex-differentially expressed as a consequence of *dsx* action, but detected few, if any, genes regulated by *fru*.

Given our potential to detect genes sex-differentially deployed in any somatic tissue of adults, it is striking that all 11 genes whose expression patterns we determined were expressed in internal genital tissues. Although we have only assayed 11 out of 63 potential genes here, the results of previous, smaller scale screens are consistent with our results (reviewed by Wolfner, 1988). The tissues that comprise the internal genitalia are small; the largest is the male accessory gland, which has about 1050 cells (Bertram et al., 1992). Thus our experiments could readily detect sex-differential gene expression in a very small fraction of adult cells, with the qualification that, to the degree the tissues of the internal genitalia are producing large amounts of a limited number of transcripts, this sensitivity may be less impressive than it seems.

Why, then, as we know that cells of other parts of adults are sexually dimorphic, did whole organism studies not detect genes expressed sex-differentially in these tissues? Whole-animal assays are probably missing these genes because they are sex-differentially expressed at low levels in relatively few cells, or sex-specifically in one tissue and sex-nonspecifically in other tissues. These considerations are supported by a comparison of the genes identified by SAGE analysis as sex-differentially expressed in the head (Fujii and Amrein, 2002) with our data set. Of the 19 genes identified as having significant sex-differential expression levels in head tissue, five were in our array (Cypd4d21, CG4979, CG11458, CG7433, CG5288). Cypd4d21 showed significant sex-differential expression, in the same direction, in both studies. By contrast, CG7433 expression was significantly higher in male head tissue, but significantly lower in male whole bodies in our study. We did not detect a sex difference in the expression levels of the other three genes. These considerations probably explain why genes differentially expressed as a consequence of *fru* activity were not identified.

Regulation by the sex hierarchy of sex-differentially expressed somatic genes

Temporal regulation of sex-differential adult gene expression by the hierarchy

We next examined when sex hierarchy regulation of the 11 selected genes occurs. There are two known mechanisms by which sex-differential gene expression in adults is generated: (1) the sex hierarchy actively regulates gene expression in adults, as is the case for *Yolk protein 1* (*Yp1*) (Belote et al., 1985); or (2) the hierarchy functions earlier in development to specify which sex-specific adult tissues will be formed but does not regulate gene expression in those tissues in the adult (reviewed by Christiansen et al., 2002). We used temperature-sensitive *tra2* alleles, which allowed us to switch between the male and female mode of splicing *dsx* (Nagoshi et al., 1988). In chromosomally XX *tra-2*^{ts} animals, female development occurs at the permissive temperature (16°C), whereas male development occurs at the non-permissive temperature (29°C). Animals were raised at one temperature, collected 0-24 hours after eclosion and maintained at their original temperature for one more day. We then switched half of each group to the other temperature (16°C to 29°C, or 29°C to 16°C). All animals were maintained for three more days, and RNA was then extracted. Under these conditions, expression of *Yp1* (the positive control, Fig. 6C) responded to temperature shifts as expected (Belote et al., 1985); the *Yp1* transcript was reduced when animals were switched from 16°C to 29°C and induced when animals were switched from 29°C to 16°C. By contrast, expression of the 11 other genes analyzed did not change substantially over the three days following the temperature shifts (Fig. 6). Thus, sex-differential expression of all 11 genes is the consequence of the developmental action of the sex hierarchy and is independent of the hierarchy during adult stages.

As all 11 of the genes we analyzed in these *tra-2*^{ts} experiments are expressed within the sex-specific genital organs, these findings have implications for how the sex hierarchy functions in the construction of sex-specific organs. In the male accessory gland and ejaculatory duct, the sex hierarchy has been shown to function during the late larval/early pupal period to specify the adult expression patterns of several genes (DiBenedetto et al., 1987; Chapman and Wolfner, 1988; Feng et al., 1991). Our results for CG18284, CG17022 and CG17843, which are expressed exclusively in the male accessory glands, and for CG8708, which is expressed in the ejaculatory duct, are consistent with these findings in that the four genes are regulated by the action of the sex hierarchy prior to adulthood. For three other internal genital organs, the ejaculatory bulb, spermathecae and parovaria, there was no prior knowledge about when the hierarchy functioned. Our data indicate that the adult sex-specific patterns of expression of at least some genes in these tissues (CG2858 in ejaculatory bulb, and CG17012 in spermathecae and parovaria) are also determined by the prior developmental action of the hierarchy. Taken together, these parallel findings with respect to five of the major organs of the internal genitalia are consistent with the notion that the sex hierarchy functions developmentally in all these organs to set up the eventual patterns of adult gene expression.

Modes of dsx regulation of sex-differentially expressed genes

As suggested by our microarray data, the 11 genes expressed in internal genital organs are almost certainly regulated by *dsx* rather than by *fru*, as *dsx* is known to regulate development of these tissues and FRU^{M} is not expressed within the internal genitalia. The DSX^{M} and DSX^{F} proteins are known to have both positive and negative roles (Baker and Ridge, 1980). We therefore sought to understand the manner in which the DSX proteins regulate these genes. For the two genes expressed in multiple tissues in adults, we carried out in situ hybridizations to XX and XY *dsx* null individuals to assess the mode of *dsx* regulation in individual organs. For the five genes that are expressed in one organ of the internal genitalia and nowhere else in adults, we used microarray data from *dsx* null individuals. The results of these analyses are suggestive of multiple modes of *dsx* regulation.

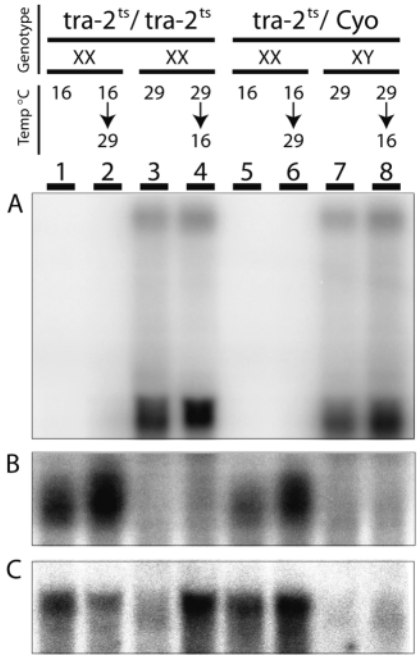


Fig. 6. Characteristic examples of RNA blot analyses for *tra2* temperature-shift experiments. Total RNA (20 mg) was derived from *tra2*^{ts1}/*tra2*^{ts2} (lanes 1-4) animals and *tra2*^{ts}/*Cyo* controls (lanes 5-8); RNA in lanes 1-6 was from animals that were chromosomally XX, whereas and RNA in lanes 7 and 8 was from animals that were chromosomally XY. Animals were either maintained at one temperature, or raised at one temperature then shifted as 2-day adults to the other temperature, as indicated. (A) CG18284, a male gene encoding two transcripts; (B) CG7777, a female gene; and (C) *Yp1*.

To determine how *dsx* regulated the female gene CG17012 in spermathecae, we carried out in situ hybridization on wholemounts of internal genitalia of *dsx* null and wild-type individuals. Spermathecae are not recognizable in all *dsx* mutant individuals (Hildreth, 1965), so just those individuals with spermathecae were evaluated. Reduced expression, as

Table 3. Modes of regulation by *dsx*

Gene	Expression localized to	Genotype / DSX phenotype: XY wt DSX^{M}	XX wt DSX^{F}	XY *dsx* –	XX *dsx* –	XX *dsx*^{D} DSX^{M}	XX *tra* DSX^{M}	Suggested mode of regulation: DSX^{M}	DSX^{F}
*Yp1**	Female fat body							–	+
CG17012	Spermathecae	–1.29	4.64	–0.95	–0.15	–1.34	–1.89	0	+
CG17843	Male AG	1.31	–3.16	1.08	0.09	0.80	0.55	0	–
CG17022	Male AG	3.29	–2.69	3.83	2.28	3.86	3.75	0	–
CG18284	Male AG	2.92	–1.60	2.46	0.43	3.33	2.39	0	–
prd	Male AG	0.40	–0.30	0.62	0.61	1.31	0.94	0	–
CG2858	EB	1.10	–2.07	–0.68	0.35	1.24	1.01	+	–
CG8708	AED	1.15	–1.07	0.50	0.05	1.79	2.00	+	–

Values given in table are log-transformed microarray ratios.
*Mode of regulation previously established (reviewed by Christiansen et al., 2002).
AG, accessory gland; EB, ejaculatory bulb; AED, anterior ejaculatory duct; wt, wild type; 0, no effect; +, positive effect; –, negative effect.

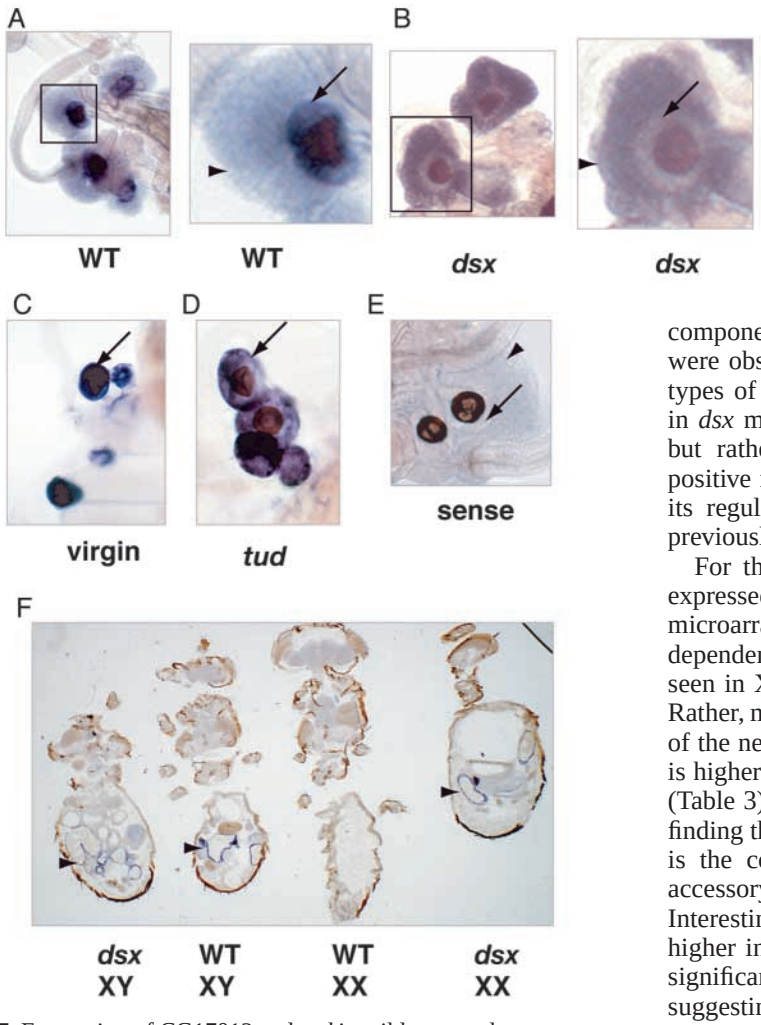


Fig. 7. Expression of CG17012 and *prd* in wild-type and mutant tissues from 5-day-old flies. (A-D) Whole-mount in situ analysis of CG17012 expression in female internal genitalia in: (A) wild type, (B) *dsx* intersexual animals, (C) virgin wild type, and (D) *tud* females. In A and B, the region within the box has been digitally magnified and shown on the right. Staining is observed in cells of the spermathecae and in the fat cells that surround the spermathecae in A, but only in the fat cells in B. Fat tissue is not visible in C and D. (E) CG17012 sense probe (negative control). Low level signal was observed in fat tissue, a common occurrence with both sense and anti-sense probes. Arrows indicate cells of the spermathecae and arrowheads indicate fat cells. (F) Whole animal frozen section in situ analysis of *prd* expression in *dsx* intersexual and wild-type animals. Arrowheads indicate expression regions

compared with wild-type females, was seen in spermathecae of *dsx* null individuals (Fig. 7A,B). The diminished CG17012 expression in *dsx* mutants suggests that DSX^F positively regulates its sex-specific expression. The microarray data for CG17012 are consistent with this conclusion and do not reveal any effect of DSX^M on CG17012 expression. However, another possible explanation for reduced expression in *dsx* null individuals is that *dsx* mutants are unable to mate. There is evidence for changes in female behaviors mediated (perhaps at the transcriptional level) by seminal fluid and sperm transferred during copulation, including a reduction in receptivity to courtship and enhanced egg laying (reviewed by Wolfner, 2002).

To determine whether copulation, seminal fluid or sperm affect expression of CG17012, whole-mount in situ hybridization was performed on tissue from the following 5-day-old adults: (1) wild-type females; (2) virgin wild-type females that were separated from males prior to eclosion; and (3) *tud* females mated to *tud* males, which do not produce sperm (Boswell and Mahowald, 1985) but transfer other seminal fluid components. Comparable high levels of CG17012 expression were observed in the spermathecae and parovaria of all three types of females (Fig. 7A,C,D), suggesting lower expression in *dsx* mutants is not a consequence of their failure to mate, but rather that female-specific expression is the result of positive regulation by DSX^F, and that DSX^M plays no role in its regulation. This pattern of *dsx* regulation has not been previously reported.

For the three genes (CG17843, CG17022 and CG18284) expressed exclusively in the male accessory glands, the microarray results suggest that their expression is not dependent on DSX^M, as comparable high expression levels are seen in XY wild-type and XY *dsx* null individuals (Table 3). Rather, male-specific expression appears to be the consequence of the negative action of DSX^F in females, as their expression is higher in XX *dsx* null individuals than in wild-type females (Table 3). These observations are consistent with the previous finding that male-specific development of the accessory glands is the consequence of DSX^F acting in females to prevent accessory gland formation (Ahmad and Baker, 2002). Interestingly, expression of these three genes is significantly higher in XY *dsx* null than XX *dsx* null ($P<0.05$), but is not significantly different between wild-type males and *fru* males, suggesting an additional sex-differential complexity to their expression.

prd is also expressed in the male accessory gland, and is of interest as its expression is required during both development and adulthood for accessory gland formation and physiology (Xue and Noll, 2002). We examined the role of *dsx* in *prd* expression by means of in situ hybridization of frozen sections of XX and XY *dsx* null individuals, and XX and XY wild-type controls. Comparable levels of *prd* expression were observed in the accessory glands of XY wild-type animals and both XX and XY intersexual animals, with no visible expression in XX wild-type animals (Fig. 7F). These observations suggest that male-specific expression of *prd* is not a consequence of positive regulation by DSX^M, but rather of negative action by DSX^F to prevent the formation of male accessory glands, as was observed for the other accessory gland genes above.

Finally, for CG2858 and CG8708, expressed exclusively in the ejaculatory bulb and ejaculatory duct, respectively, the microarray data suggest another mode of *dsx* regulation. For both of these genes, expression appears to be lower in XY *dsx* null animals than in the three genotypes expressing DSX^M (XY wild type, XX *tra* and XX dsx^D pseudomales, Table 3), suggesting that DSX^M positively regulates their expression. Expression also appears higher in XX *dsx* null compared with XX wild-type animals, suggesting negative regulation by DSX^F in the formation of these tissues in females. This pattern of regulation has not been previously reported for a gene under

the control of *dsx*, although it is the exact converse of how the *Yp* genes are regulated.

Taken together these findings reveal a rich diversity of *dsx* function. In the accessory gland the sole role of *dsx* revealed to date is the action of DSX^F to prevent the formation of the organ in females, whereas in the spermathecae, ejaculatory duct and ejaculatory bulb *dsx* appears to have two types of functions. First, the hierarchy must be acting, via *dsx*, to direct these tissues to an alternative developmental fate in the inappropriate sex (Hildreth, 1965; Keisman et al., 2001). Second, as shown here, *dsx* may also function in the appropriate sex in these three organs prior to adulthood, and probably during the late larval/early pupal period (DiBenedetto et al., 1987; Chapman and Wolfner, 1988; Feng et al., 1991), to establish the potential for the appropriate patterns of gene expression.

The sex determination hierarchy in *Drosophila* is well understood at the molecular-genetic level, but the genes that are sex-differentially regulated by the hierarchy have only begun to be identified. Here, we examined sex-differential gene expression in adults, the stage of the *Drosophila* life cycle that displays the most striking differences between the sexes. This study adds substantially to our knowledge of the types of genes expressed sex-differentially in somatic tissues, provides molecular entry points for elucidating the functions of reproductive organs of both sexes, and expands our understanding of the timing and mode of gene regulation by the sex hierarchy.

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