AN ABSTRACT OF THE THESIS OF

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Barbara J. Taylor

To determine the role of *doublesex* (*dsx*) in the regulation of the development of sex-specific musculature, we have examined the development of a set of sexually dimorphic genital muscles. In both adult males and females ten muscles attach to the genitalia and terminal segments in sex-specific patterns. Six of these genital muscles in males and seven in females consistently express ß-galactosidase from a *P[79Bactin-lacZ]* construct.

XY and XX *dsx* mutants that develop as intersexes possess both male and female genitalia. In both XX and XY *dsx*$^-$, and XX *dsx*-dominant, intersexes, we find the same subset of male and female genital muscles. Unlike muscle staining in wildtype flies, staining for ß-galactosidase in the intersexes is irregular, suggesting that expression of the *P[79Bactin-lacZ]* construct is a separate phenotype from muscle presence. In total, we find approximately nine genital muscles in the *dsx* intersexes, similar to the number of muscles found in either male or female wildtype flies. The

failure of nearly half of the possible male and female genital muscles to form may be due to the absence of appropriate attachment points on the cuticle or to a limiting number of muscle precursor cells. From the similar pattern of muscles in the two different types of dsx mutant intersexes, we conclude that *dsx*$^{+}$ function directs the development of the genital muscles, acting in wildtype flies to repress the development of muscles of the inappropriate sex.

Lastly, I describe a set of putative myoblasts that are likely candidates for the precursors of the genital muscles. A similar set of putative myoblasts is found in male, female and intersexual discs, suggesting that the myoblasts act as a single primordia for the genital muscles.

Regulation of the Development of Sex-Specific Genital Muscles by the *doublesex* Gene

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Thomas J. S. Merritt

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APPROVED:

Redacted for Privacy

Major Professor, representing Zoology

Redacted for Privacy

Chair of Department of Zoology

Redacted for Privacy

Dean of Graduate School

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Chapter 1
An Introduction to Sex Determination and Muscle Development in *Drosophila melanogaster*

General overview of thesis

Development of sex-specific structures and behaviors is an attractive system in which to investigate the genetic mechanisms that control adult differentiation. Sex-specific structures are not needed for individual viability, therefore, mutations that cause sexual transformation can be studied throughout the life cycle. Further, genetic and molecular studies have shown that, in *Drosophila melanogaster*, sex-determination is a function of a relatively small number of genes, making *Drosophila* a particularly attractive system in which to study the genes involved in establishing a male or female developmental pathway for sexually dimorphic tissues (recent reviews: Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Burtis and Wolfner, 1992; Burtis, 1993).

I have investigated the role of one of the sex-determining genes, *doublesex* (*dsx*), in controlling the development of a set of sex-specific muscles in *Drosophila melanogaster*. The muscles I have studied surround and/or attach to the internal and external genitalia in a stereotypic, sex-specific pattern. During the metamorphosis from the larva to the adult, larval muscles histolyse and adult skeletal muscles arise *de novo* from muscle precursor cells set aside in the embryo. The final morphology and location of the genital muscles must then depend on the activity of multiple genes that, as a group, regulate both muscle differentiation and sexual differentiation.

In the first half of this chapter I describe sex-specific differences in *Drosophila* and give an overview of the genetic control of the development of these characteristics. In the second half of this chapter I review muscle development in *Drosophila*. In the second chapter, I describe, in detail, the genital muscles in wildtype and *dsx* mutant flies and discuss the role of *dsx* in development of these muscles. In the third chapter, I briefly describe the location of putative myoblasts in the genital disc, a possible source of the genital muscles. In the fourth, and concluding, chapter, I speculate on future directions for this research.

Description of sexually dimorphic characteristics

The two sexes of *Drosophila* differ in many external and internal structures (Ferris, 1950; Miller, 1950), and in the production of sex-specific molecules, such as pheromones and yolk proteins (see, for example, Burtis and Wolfner, 1992; recent reviews: Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Burtis and Wolfner, 1992; Burtis, 1993). Furthermore, each sex displays specific courtship and mating behaviors (see, for example, Taylor et al., 1994). The adult sex-specific differences are first visible in larvas as growth differences in imaginal tissues. Final elaboration and differentiation of these sex-specific imaginal tissues occurs during metamorphosis. Sex-specific differences in the germ line and its control, while of great import to the individual and the species, will not be addressed here (but, see for recent review, Steinmann-Zwicky, 1992; Gorman and Baker, 1994).

The body of an adult fly is divided into three distinct regions: head, thorax and abdomen. Most sex-specific differences are associated with the abdomen, but sets of sex-specific chemo- and mechano-sensory bristles are present on the head and forelegs. *Drosophila* have six legs, each leg has ten segments: the coxa, trochanter, femur, tibia and five tarsal segments. On the first tarsal segment of the prothoracic (most anterior) leg, males have a row of large, right-angled, blunt, heavily pigmented bristles, known as the sex comb (Ferris, 1950; Tokunaga, 1962). In females the same tarsal segment has two rows of bristles, identical in size, angle and pigmentation to bristles on the other legs. Additionally, along the entire prothoracic leg the number and organization of gustatory bristles is also sex-specific; males have a greater number of bristles than females (Possidente and Murphey, 1989). Cranially, and finally, while not as outwardly sexually dimorphic as in some insects, *D. melanogaster* antenna do show sex-specific distributions of chemosensilla (Stocker and Gendre, 1988).

The adult abdomen is segmented; most segments have a dorsal cuticular plate, the tergite, and a ventral cuticular plate, the sternite. Abdominal sexual dimorphisms include the number of segments, segment pigmentation, and, most posteriorly, the genitalia and analia. Males have six large, serially repeated, abdominal segments (A1-A6); females have seven (A1-A7). In males, the tergites of the fifth and sixth segments are uniformly pigmented, completely dark. In females, all tergites are only pigmented across their posterior region, producing a banded pattern. The genital and anal structures are located at the caudal end of the abdomen.

These anal and genital structures are the most dramatic of the sexual differences. Two laterally paired, equal-sized, cuticular plates cover the anus of male flies, in females this pair of anal plates is dorsal-ventral, with a larger dorsal, than ventral, plate. Centrally, the penis is located within the penis apparatus, ventral to the anal plates. The external male genitalia includes the genital arch, lateral plates and the hypandrium, these all surround the anal plates and penis apparauts. In females, a small dorsal eighth tergite surrounds the anal plates and extends to either side of the two cuticular structures covering the vulva, the vaginal plates. Both male and female analia and external genitalia are covered with bristles in specific patterns (Taylor, 1992).

The genitalia and analia develop from a single imaginal disc, the genital disc, attached along the ventral midline in the posterior of both male and female larvae. Gynandromorph and somatic recombination studies (Nöthiger et al., 1977; Schupbach et al., 1978; Epper and Nöthiger, 1982) as well as metamorphosis of disc fragments (Epper, 1981; Epper, 1983a; Epper and Bryant, 1983), have shown that this imaginal disc contains three separate primordia, a male primordium, a female primordium and an anal primordium, in third instar larvae of either sex. Sex specific cell growth, and death, during metamorphosis results in the final sex-specific adult phenotypes of the genitalia and analia (Epper, 1983b; Taylor, 1989a).

The male primordium is in the anterior and lateral region of the disc; in males this region differentiates into the set of internal and external structures that make up the male reproductive system (Epper and Nöthiger, 1982; Taylor, 1989a). In females,

the cells of this region divide very slowly during larval life and eventually die during metamorphosis (Epper and Nöthiger, 1982; Epper, 1983b; Taylor, 1989a). The female primordium occupies the ventral region of the disc, in females it develops into the external and internal genitalia; in males this region degenerates (Epper and Nöthiger, 1982; Epper, 1983b; Taylor, 1989a). The single anal primordia is located the dorsal posterior-most region of the disc. In females the anal primordia gives rise to the dorsal and a ventral anal plates. In males the anal primordia, or a subset of this region, gives rise to a pair of lateral anal plates (Epper and Bryant, 1982; Taylor, 1989a).

Internal sexual dimorphisms have been described in the musculature, nervous system and fat body. Given the pronounced differences in the external genitalia it is not surprising that there are different genital muscles in males and females. These muscles have previously been incompletely cataloged in descriptions of adult abdominal cuticle and musculature (Ferris, 1951; Miller, 1951). In the next chapter I give a more complete description of these genital muscles. To date, only one other sex-specific muscle has been reported, the Muscle of Lawrence (MOL), found in the fifth abdominal segment of adult males, but not adult females (Lawrence and Johnston, 1984). This bilaterally paired muscle lies lateral to the dorsal midline, spanning nearly the full extent of the fifth abdominal segment.

As required by their respective reproductive tasks, males and females perform distinctive behaviors, (for review, Speith, 1974). Males have a behavioral repertoire associated with precopulatory and copulatory events. Females, on the other hand,

accept or reject courtship, engage in copulation and lay eggs on suitable substrates. While control of these behaviors has been mapped to the CNS using gynandromorphs (Hall, 1977; Hall, 1979; Tompkins and Hall, 1983), only a few specific neuroanatomical sexual dimorphisms have been described. Sex-specific nerve terminals in the central nervous system are associated with the sex-specific sensory bristles described above (Possidente and Murphey, 1989; Taylor, 1989b). Additionally, a higher order olfactory processing center, the mushroom body, has a different number of fibers in males and females (Technau, 1984). However, no role in courtship has been demonstrated for this brain region; males with severe mushroom-body defects still court in an apparently normal fashion (Heisenberg, 1980; deBelle and Heisenberg, 1994). Instead these regions appear to be involved in olfactory learning (deBelle and Heisenberg, 1994), a reason for the sex-specific morphology has not been found. Another sex-specific difference has been described in a dozen abdominal neuroblasts. Male neuroblasts have more divisions during larval and early pupal stages than female neuroblasts, resulting in upwards of 20 additional neurons per neuroblast in an adult male than an adult female (Taylor and Truman, 1992). But, again, no specific function for these neurons has been described.

Males and females also differ in some of the biochemical products they produce. Among the compounds specifically made by males are those made by the male accessory gland, an internal genital organ derived from the male primordia of the genital disc, and transferred to females during mating; several of the proteins act to

alter subsequent behaviors by inseminated females (see, for example, Kubli, 1992). Females produce long-chain hydrocarbons that act as mate attractants, these molecules are produces in much smaller quantities by males (for review of chemical communication in *Drosophila* see Jallon, 1984). In *Drosophila*, some of the proteins that make up the egg yolk are produced in the female fat bodies and transported to the gonads, and developing eggs, through the hemolymph. Male fat bodies do not normally produce yolk proteins (Ota et al.,1981; Coshigano and Wensink, 1993; reviewed by Bownes, 1994; Burtis and Wolfner, 1992). Further, a set of male-specific RNA's, the male-specific transcripts (*mst*'s) are produced by the male accessory gland (DiBenedetto, 1987). The *glucose dehydrogenase* gene (*Gld*) is also expressed in a sex-specific pattern during development of both male and female genitalia (Feng et.al., 1991).

General model for somatic sexual differentiation

The somatic differentiation of *Drosophila melanogaster* into either a male or female morphology depends on the activity of a gene cascade transmitting information on the chromosome complement to downstream genes responsible for sex-differentiation (Figure 1; recent reviews: Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Belote, 1992; Burtis and Wolfner, 1992; Cline, 1993). Female *Drosophila* are homogametic, XX, males are heterogametic, XY. Unlike the situation in mammals, it is the ratio of X chromosomes to autosomes (X:A

ratio) that determines sex, not the presence or absence of a sex-determining chromosome (Bridges, 1921; Cline, 1983; 1988; reviewed by Hodgkin, 1992; Cline 1993; Gormanand Baker, 1994). Females, with two X chromosomes and a diploid set of autosomes, have a X:A ratio of 1; males, with only a single X have a ratio of .5.

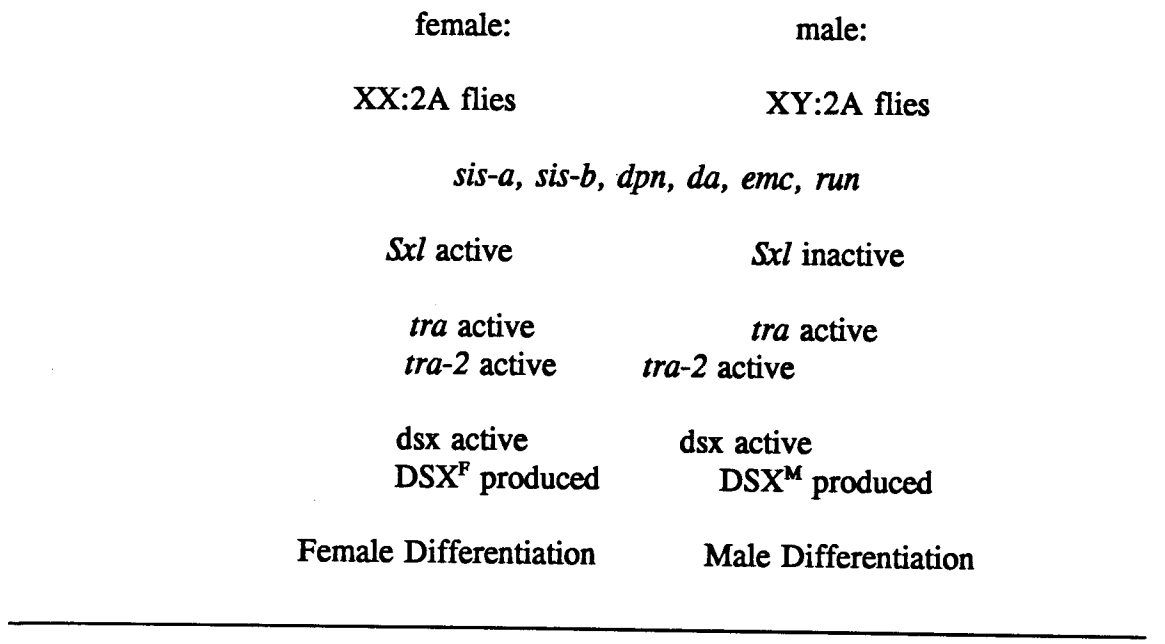

Figure 1 Summary of the sex-determination gene cascade.

In the somatic tissues, decisions on sexual fate are predominantly cell-autonomous. This autonomy is apparent in mosaic flies in which the soma is a mix of both chromosomally male (X0) and chromosomally female (XX) cells (eg Nöthiger et al., 1977). Individual cells express the sexual fate, male or female, appropriate for their

chromosomal state, not that of their neighbors. The fifth segment male-specific muscle, the MOL, is an important exception to this cell-autonomous control of sex-determination (Lawrence and Johnston, 1986), as will be discussed later.

Control of sex-determination in Drosophila can be likened to a series of on/off switches: on, female differentiation; off, male differentiation (reviews: Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Belote, 1992; Burtis and Wolfner, 1992; Cline, 1993). Each switch is set by the presence, or absence, of a functional product from the previous gene in the cascade. The product of one gene influences the pattern of splicing of the next gene in the cascade, so that a common pre-mRNA is differentially spliced to yield sex-specific transcripts. These sex-specific transcripts are then translated into functional proteins, in one, or both, of the sexes, which then influence the splicing of the next gene. In most cases the outcome of differential splicing produces functional protein in only one sex, but at the output level of the cascade both spliced products yield active protein products.

The gene cascade consists of five known genes; *Sex-lethal (Sxl), transformer (tra), transformer-2 (tra-2), doublesex (dsx)* and *intersex (ix)*. In female *Drosophila*, an active, female-specific, *Sxl* gene product is produced (Figure 1). This protein promotes female-specific splicing of the *tra* primary transcript. The female-specific transcript is then translated into an active, female-specific, TRA protein. The *tra-2* protein, TRA, is found in both sexes. In females, the TRA and TRA-2 proteins act together to control splicing of the *dsx* primary transcript. The female-specific *dsx* protein, DSX^F, along with the product of the *ix* gene, represses male differentiation

and promotes female differentiation. In males, where no active *Sxl* protein is produced, the *tra* pre-mRNA is spliced in a default pattern that does not encode a protein. In the absence of TRA protein, *dsx* pre-mRNA is spliced into the male-specific mRNA. In contrast to the male-specific *Sxl* and *tra* transcripts, male-specific *dsx* mRNA encodes a functional protein, DSX^M, that is required for normal male development.

The *Sex lethal* gene

At the top of the genetic cascade, *Sxl*, regulates dosage compensation, as well as somatic and germline sex-determination. To maintain equal levels of X chromosome gene products in both sexes, transcription from the single X in males is boosted to twice the level of a either X in females (reviewed by Hodgkin, 1992; Gorman and Baker, 1994). The SXL protein prevents this hypertranscription in females. Null mutations in *Sxl* are generally female lethal due to a failure to prevent hypertranscription of the X-chromosomes (Marshal and Whittle, 1978; Cline 1980; 1983). When the sex-determining function of *Sxl* is disrupted, without destroying its dosage compensation function, XX flies develop as somatic males (Cline, 1984). XY *Sxl*$^-$ flies develop as normal males, in fact, *Sxl* activity is deleterious to males, preventing the hypertranscription of their single X chromosome (Marshal and Whittle, 1978; Cline 1980; 1983).

Sxl activity is initially directed by transcriptional activation early in development (Salz et al., 1989; Keyes et al., 1992). The *Sxl* gene has two promoters, an early

promotor, P_E and a late promotor, P_L. Early production of *Sxl* protein appears to be a function of use of the stage- and sex-specific promotor, P_E. Transcripts from P_E are spliced in a female-specific pattern, leading to the functional protein. Transcription is initiated from P_E only in females and only during a brief interval during the syncytial blastoderm stage. The mechanism restricting the use of P_E is as yet incompletely understood, but one model for *Sxl* activation, involving both maternal and zygotic factors, seems to best fit the currently available data (Cline, 1988; 1993; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Belote, 1992). In this model the ratio of certain X-linked genes, numerator genes, to some autosomal genes, denominator genes, determines the activity of the *Sxl* gene. This ratio is transmitted to the *Sxl* gene by a set of maternally and zygotically transcribed gene products. Mutations in genes acting as either numerators or denominators would result in errors in dosage compensation, which, in turn, would result in zygotes of only one sex. Two genes, *sisterless-a* and *sisterless-b (sis-a, sis-b)*, have been identified as numerator genes (Cline, 1988). Whereas the gene *deadpan (dpn)* appears to be the only denominator gene (Younger-Shepherd et al., 1992).

Sex-lethal activity also requires the maternal function of at least two other genes, *daughterless* (*da*; Cline, 1980; 1983) and *extramacrochaete* (*emc*; Younger-Shepherd et al., 1992). While *da* and *emc* are required for proper sex-determination and dosage compensation, they are not required in a dosage specific manner. They are not, therefore, numerator or denominator genes, but, rather, function through some other role, possibly in transmitting the numerator:denominator ratio to *Sxl*.

Zygotically transcribed *runt* gene product is also required in a non-dosage dependent manner for proper *Sxl* activity (Torres and Sanchez, 1992).

Molecularly, control of *Sxl* activity is thought to involve protein-protein interactions which titrate certain DNA binding proteins. *sis-b, dpn, da* and *emc* all code for proteins with sequences consistent with a helix-loop-helix (HLH) secondary structure (Younger-Shepard et al., 1992). This structure is characteristic of proteins that bind DNA as hetero- or homo-dimers (Murre et al., 1989). The activity of these proteins may be a function of their binding to the *Sxl* gene and/or each other.

Following the syncytial blastoderm stage, in both sexes transcription shifts from the from the P_E promoter to the P_L promotor. This later transcription only leads to production of functional protein in females. In females a *Sxl* transcript is present that does not contain the third exon and does contain a long ORF (Bell, 1988). This transcript codes for a protein similar to that produced from the P_E transcription product. In males all *Sxl* primary transcripts, all transcribed from the P_L promotor, contain a third exon containing multiple stop codons. These transcripts do not contain any long ORF and no *Sxl* protein is produced (Bell, 1988).

An autoregulatory mechanism ensures that functional *Sxl* protein continues to be made in females; once active *Sxl* protein has been produced, from P_E during normal female development, it is both necessary and sufficient to regulate the continued production of active *Sxl* protein, irrespective of future chromosome complement. SXL binds to its own transcript, promoting female-specific splicing (Bell et al., 1991; Sakomoto et al., 1992). This binding is dependent on multiple uridine rich sequences

found around the male-specific exon. Deletion of these sequences eliminates female specific splicing. Female-specific splicing was restored with addition of new poly-U sequences to the deleted constructs. Similar poly-U sequences are important in Sxl regulated sex-specific splicing of the *transformer* primary mRNA.

Sex-lethal regulation of the transformer gene

transformer is the next gene in the sex-determination regulatory cascade (Baker and Ridge, 1980, Nagoshi et al., 1988). Active *tra* gene product is required for proper female sexual differentiation; XX *tra*$^-$ flies develop as phenotypic males (Sturtevant, 1945). Active TRA is not required in males; XY *tra*$^-$ homozygotes develop as normal males (Baker and Ridge, 1980).

Production of TRA protein is dependent on sex-specific splicing of a non-sex-specific *tra* pre-mRNA into sex-specific transcripts (Boggs et al., 1987; Mckeown et al., 1988). This splicing is controlled by SXL binding to specific sequences within the *tra* pre-mRNA in a manner similar to that in which SXL controls splicing of its own transcript (Sosnowski et al., 1989; Inoue et al., 1990; Valcarcel et al., 1993). Thus, two *tra* mRNA transcripts are found in adult *Drosophila*. These transcripts vary in the first exon splice acceptor site used; use of the upstream site produces a mRNA found in both sexes. Whereas use of the downstream site produces a mRNA only found in females (Boggs et al., 1987). Only this female specific transcript contains a long ORF and produces functional *tra* protein (McKeown et al. 1988).

Potentially, Sxl could control splicing of *tra* pre-mRNA by either promoting the use of the 3', female-specific, splice acceptor site or blocking the use of the 3', non-specific, site. Tests of the site of SXL action showed that deletion of the non-sex-specific splice site leads to *Sxl*-independent use of the female-specific splice site *in vivo* (Sosnowski et al., 1989). *In vitro*, the *Sxl* protein binds specifically to poly-U sequences around the non-specific splice site (Inoue et al., 1990, Valcarcel et al., 1993), similar to the poly-U sequences near exon 3 of the *Sxl* transcript that are involved in *Sxl* self-regulation (Sakamoto et al., 1992). Binding of SXL to these sequences prevents formation of the spliceosome at the non-specific splice acceptor site and the female-specific site is used by default (Valcarcel et al., 1993). This strongly suggests that *in vivo Sxl* mediated control of *tra* splicing is a function of the SXL protein binding directly to the splice acceptor site within the non-sex-specific *tra* exon blocking its use and preventing inclusion of this exon in the *tra* final transcript.

The *transformer-2* gene

tra-2 loss-of-function mutants have a phenotype very similar to *tra* loss-of-function mutants (review, Baker and Belote, 1983). The *transformer-2* protein acts, along with the product of the *transformer* gene, to promote female-specific splicing of the *doublesex* primary transcript (Baker and Ridge, 1980; Belote and Baker, 1982; Nagoshi et al., 1988). Like the TRA protein, the TRA-2 protein is required continuously in the female soma for normal sexual development, but is not required for normal male somatic development (Belote and Baker, 1982). Unlike the TRA

protein, the TRA-2 protein is required for proper spermatogenesis in the male germ line (Belote and Baker, 1982). A functionally similar TRA-2 protein is found in the soma of both sexes, but influences *dsx* pre-mRNA splicing only in the presence of functional TRA protein.

Molecular evidence reveals a possible mechanism of action. The TRA-2 protein shows noted sequence homology to a family of RNA binding proteins that includes hnRNPs and snRNPs, suggesting that *tra-2* may act by binding directly to the *dsx* transcript (Goralski et al., 1989).

The *doublesex* gene

Both genetic (Baker and Ridge, 1980) and molecular (Nagoshi et al.,1988) studies place the *doublesex* gene (Hildreth 1965) downstream of *Sxl, tra* and *tra-2*. *Sxl, tra* and *tra-2* are all required for proper *dsx* activity in females; *Sxl* acts trough *tra*, which along with *tra-2* acts directly on *dsx*. *dsx* is the only sex-determining gene which is required for normal somatic sexual development of both sexes; both XX or XY dsx^- homozygotes develop as intersexes, expressing a combination of male and female sex-specific characteristics (Hildreth, 1965; Baker and Ridge, 1980).

The general theme of control through sex-specific splicing is seen in the control of *dsx*. Late in larval development male- and female-specific transcripts first appear through the alternative splicing and polyadenylation of a common pre-mRNA which produces transcripts with identical 5' ends, but different 3' ends (Baker and Wolfner, 1988; Burtis and Baker, 1989). Exons 1,2,3 are found in transcripts from both sexes.

Exon 4 is found only in female-specific transcripts, while exons 5 and 6 are found only in male-specific transcripts. These transcripts are translated into sex-specific proteins with identical carboxy-, but different amino-, terminus, DSX^M and DSX^F.

Production of the female-specific transcript is the regulated step in control of *dsx* activity which is controlled by the activity of the *tra* and *tra-2* genes. In the absence of either *tra*, or *tra-2*, as in males or *Sxl*$^-$, *tra*$^-$ or *tra-2*$^-$ females, the *dsx* pre-mRNA is spliced in the male pattern by default. Production of the female-specific transcripts could be regulated through control at either a splicing event or at the choice of polyadenylation sites. Multiple investigations of both the splice site and the polyadenylation site point towards a regulated splicing event. There are four lines of evidence supporting the regulation of female-acceptor site by TRA and TRA-2 interaction. The female-specific fourth exon splice acceptor site shows poor homology to the *Drosophila* consensus splice acceptor site sequence, while the male exon slice acceptor site shows a good consensus, supporting the possibility that control could be via discrimination between the two sites (Burtis and Baker, 1989). Interestingly, a number of dominant mutations have been isolated that effect normal female sexual development, but do not effect normal male sexual development (Feng and Gowen, 1957; Baker and Ridge, 1980; Nöthiger et al., 1980). XX flies with a dominant *dsx* allele over a *dsx* deficiency (dsx^{Dom}/dsx^-) develop as somatic males; XY dsx^{Dom}/dsx^- flies develop as normal males. The aberrations causing these dominant *dsx* alleles are all located around the female-specific splice site and distant from the polyadenylation signal sequence (Baker and Wolfner, 1988; Burtis and Baker, 1989;

Nagoshi and Baker, 1990), further implicating splicing and not polyadenylation as the point of control. Finally, the female-specific fourth exon contains six copies of a 13 nucleotide repeat (Nagoshi and Baker, 1990). All of the dominant *dsx* alleles delete or displace the region containing these six repeats (Nagoshi and Baker 1990). *In vitro* binding experiments have demonstrated that these sequences are the *cis*-acting control elements for sex-specific splicing (Inoue et al., 1992). In cultured *Drosophila* cells a *dsx* "minigene", a construct containing portions of the third, fourth and fifth exons, is spliced in the female specific pattern in the presence of the TRA and TRA-2 proteins. Minigenes lacking a subset of the 13nt repeats were spliced in the male pattern, replacement of the repeats rescued female-specific splicing. Further, bacterially generated TRA and TRA-2 proteins were directly demonstrated to bind to the 13nt sequences (Inoue et al., 1992; Tian and Maniatis, 1992). Neither protein was found to bind to mutant 13 nt sequences. Similar results have been reported by other investigators showing, *in vitro*, that the 13nt repeats within the female-specific exon are both necessary and sufficient for female-specific splicing (Ryner and Baker, 1991; Tian and Maniatis, 1992). Further, and most importantly, deletion of the female polyadenylation site does not prevent female-specific splicing of the *dsx* transcript *in vitro* (Ryner and Baker, 1991). In total, this strongly suggests that in *Drosophila* regulation of female-specific splicing of the *dsx* transcript by *tra* and *tra-2* involves control of splicing, not polyadenylation.

Based on the early genetic evidence, and largely substantiated by the later molecular evidence, a model for *dsx* function was proposed in which sex-specific *dsx*

proteins acted antagonistically as repressors (Baker and Ridge, 1980; Belote and Baker, 1982). In this model each DSX protein repressed the development of characteristics specific to the other sex. If neither *dsx* product was present, neither sexual pathway was repressed, and characteristics of both sexes were expressed, an intersexual phenotype. If both products were present, as in XX; dsx^{Dom}/dsx^{+} flies, the proteins were proposed to canceled each other out, again producing intersexes. This model holds true for many of the sex-specific characteristics that have been described. As a prelude and introduction to the possible role of *dsx* in control of the genital muscles (to be discussed in the next chapter), I will review and examine the role of *dsx* in development of other sex-specific traits.

dsx control of sex-specific phenotypes

Most sex-specific phenotypes that have been studied depend on proper *doublesex* activity for their normal development. Production of either DSX^{M} or DSX^{F} determines which sex-specific phenotype will be repressed and which will develop. For example, XX flies with mutations in *Sxl, tra* or *tra-2* produce DSX^{M} (Nagoshi et al., 1988) and show a male pattern of tergite melanization, male sex combs and male genitalia (Baker and Ridge, 1980). Further, these flies show a male pattern of gustatory leg bristles and a male pattern (contralateral and ipsilateral projection) of afferent projections from these bristles (Possidente and Murphey, 1989). XX flies expressing DSX^{M} also show some male biochemical phenotypes. They express the

male-specific pattern of *glucose dehydrogenase* gene expression (Feng et al., 1991) and produce male-specific transcripts (*msts*) in their accessory glands (Chapman and Wolfner 1988).

Early studies of the effect of *dsx* mutations on sex-specific phenotypes (i.e. Baker and Ridge, 1980) suggested that *dsx* had only a negative regulatory role in development. Its role was construed as only being required for repression of sexual characteristics of the opposite sex, but for activation of the appropriate sexual phenotypes. For example, the sex-combs develop a similar intersexual phenotype in intersexes resulting from recessive mutations or dominant *dsx* mutations. The phenotype of XX $dsx^{DOM}/+$ suggests that lack of either *dsx* protein appears to have the same effect as presence of both *dsx* proteins. This is consistent with the proteins acting as repressors that can block each others activity, but would not be expected if one or both of the DSX proteins was required for normal development.

Recently, however, phenotypes have been described that do not fit with this simple repression model of activity and suggest a further, positive, regulatory role for the sex-specific DSX proteins. In female fat body cells, yolk proteins are produced and shipped to the ovaries for egg production via the hemolymph. These proteins are coded for by three genes, *yp1*, *yp2* and *yp3*. Two of these genes, *yp1* and *yp2*, are under the control of a single promotor (Barnett et al., 1980; Postlethwait and Jowett, 1980). In wild type males the fat bodies produce no yolk proteins. As would be expected from a phenotype under *dsx* control, ectopic expression of DSX^{M} in XX flies prevents *Yp* production (Ota et al., 1981). Further, DSX^{M} has been shown, *in vitro*,

to bind directly to a region of the common promotor of *yp1* and *yp2*, the fat body enhancer, FBE. Surprisingly, DSX^F also binds to the FBE *in vitro*. In fact, both DSX^F and DSX^M bind to the same three sites within the FBE (Burtis et al., 1991). It has been recently demonstrated that DSX^F is required for wild type levels of protein production in XX flies (Coshigano and Wensink, 1993). This suggest that control *in vivo* is a product of either DSX^M binding to the FBE and inhibiting transcription or DSX^F binding to the FBE and promoting transcription. How binding of these two proteins to the same region of the gene results in opposite effects is not known, but presumably results from different actions due to the differential carboxy termini of the two DSX proteins.

dsx also appears to have a positive role in control of male-specific cell division in one region of the developing CNS. As mentioned, a set of neuroblasts in the terminal ganglia show a longer period of division in male than in females (Taylor and Truman, 1992). When both DSX^M and DSX^F are present, individual neuroblasts adopt either the male or female pathway; they are apparently able to chose either the male or female sexual pathway. However, when neither protein is present, neuroblasts do not divide at all, in either sex. DSX^M is apparently necessary for any division of neurons in the male state. Control of the pattern of division, then, is not a function of repression by DSX^F, but of activation by DSX^M.

Another example of a possible positive regulatory role for DSX^M has recently been reported. Ectopic expression of DSX^M, using hsp70 promotor-*dsx* cDNA fusion products, produced a number of unexpected phenotypes (Jursnich and Burtis, 1993).

Of most interest was a transformation of leg bristles, in legs which do not normally have sex-differences, to a sex-comb-like morphology. The presence of DSX^M apparently activated expression of a sex-comb-like morphology in the legs. It was proposed that sex-comb development is controlled by a gene, or set of genes, under the control of DSX^M. In a situation similar to that of the Yp gene, but with the roles reversed, DSX^F is proposed to repress gene activity, while DSX^M acts to increase it. Obviously other genes must be involved to regulate proper leg and leg segment positioning.

Still other cases indicate that *dsx* may not be involved in sex-determination of all tissues; two examples of sex-specific characteristics that are unaffected by *dsx* mutations have been described. Female flies with loss-of-function mutations of *tra* and *tra-2*, or with mutations effecting the sex-determining functions of Sxl, perform male courtship behaviors, however, no such transformation is seen in females with loss-of-function alleles of the *dsx* or *ix* genes (McRobert and Tompkins, 1985; Taylor et al., 1994). While XX tra- or tra-2- pseudomales show male courtship, no mutations in the *dsx* gene induced XX flies to express any measurable male courtship phenotypes (Taylor et al., 1994). Similarly, while some *dsx* mutations do reduce courtship by XY flies, no *dsx* alleles ever completely eliminated courtship (Taylor et al., 1994).

The second *dsx*-independent phenotype is especially relevant to this study of sex-specific muscle development. Formation of the fifth segment male muscle, the MOL, is also independent of *dsx* or *ix* activity (Taylor 1992). Transformation of XX flies

due to mutations in *Sxl*, *tra* or *tra-2* includes expression of a MOL; transformation due to mutations in *dsx* does not. Likewise, and as with male courtship behavior, XY flies expressed a MOL regardless of mutations in *dsx*. If *dsx* is not involved, how, then, is sex-specific differentiation of these characters regulated?

Two solutions to resolve this discrepancy seem possible. Either the regulatory gene cascade branches out, above the level of *dsx* and *ix*, to include other genes, or *tra* and *tra-2* act directly to control expression in a manner similar to the activity of *dsx* on other traits (Taylor, 1992; Taylor et al., 1994). As yet, it is not possible to rule out either of these two possibilities. However, given the specific nature of the interaction between the TRA and TRA-2 proteins and the repeats in the *dsx* transcript, it seems less likely that TRA and TRA-2 would act directly on a number of different terminal differentiation genes.

One gene, which is known to control MOL development and to cause courtship defects, has been advanced as a possible candidate for a branch of the sex-determination gene cascade. Mutations of the *fruitless (fru)* locus have multiple effects on male flies (Hall, 1978; For review Hall, 1994). Along with unusual courtship behaviors *fru/fru* homozygous males lack, or partially lack, the MOL (Gailey et al., 1991). What exact role the gene(s) associated with this inversion have in patterning of the MOL has yet to be determined.

What is the role of *dsx* in determination of the genital muscles? *dsx* is not involved in determination of the MOL, the best studied sex-specific muscle leading to the question: Are the genital muscles regulated by *dsx*? We will address this question

in the next chapter. Whatever the role of the *dsx* gene, the development of the genital muscles is a function of muscle-specific regulation. Before examining the genital muscles in particular I will review muscle development in *Drosophila* in general.

Muscle development in *Drosophila melanogaster*

Drosophila are holometabolous insects; a normal life cycle includes an embryonic, three larval, a pupal and an adult stage. Larval and adult stages have distinct, and qualitatively different, stereotyped patterns of muscle underlying their epidermis, reflecting the extremely different modes of locomotion and roles in reproduction of these two stages. During the intervening pupal stage drastic changes in cuticular, muscular and nervous systems occur. This change is accomplished through both rearrangement of larval tissue and creation of adult structures *de novo* from imaginal cells.

In general adult muscles are formed *de novo* from specific pools of cells set aside in the embryo (Bate, Rushton and Currie, 1991; Bate, 1991). Larval muscles histolyse during metamorphosis, except for a set of larval muscles which function in or directly following eclosion (Crossely, 1978; Kimura and Truman, 1990). These retained larval muscles histolyse early in adult life. Additionally, larval muscles may act as a template for developing adult muscles (Shatoury, 1956; Fernandes et.al., 1991). During metamorphosis three larval thoracic muscles, the LOMs, split into six templates with which new myoblasts fuse to form six adult thoracic muscles, the

DLMs (Shatoury, 1956; Fernandes et al., 1991). The larval muscles survive the first wave of histolysis that destroys most larval muscles, split and then fuse with myoblasts set aside during embryogenesis for adult muscle formation. In at least two other insect species a similar set of muscles also develop through the fusion of myoblasts with a template formed by the remnants of larval muscle (Smit and Velzig, 1986; Cifuentes-Diaz, 1989). Interestingly, however, in *Drosophila*, at least, these templates do not appear to be required for proper muscle placement; laser ablation of the templates in developing pupae does not prevent formation of an adult muscle (Fernandes, personal communication).

Larval muscle functioning as a template for adult muscle formation, though more common in other insects, is apparently the exception in *Drosophila* (Crossely, 1978, Nüesch, 1985). While it is more common for adult *Drosophila* muscle to develop entirely from cells set aside in the embryo, this case of the LOMs and DLMs indicates that the possibility of larval templates for the sex-specific genital muscles can not, *a priori*, be ruled out. In fact, in *Manducca sexta* the genital muscles do develop from a template of larval muscle remnants (Thorn and Truman, 1989). With the onset of pupation in this species the larval terminal abdominal muscles largely histolyse and degenerate. The remaining non-contractile "scaffold" of muscle remnants forms a sex-specific template through cell loss and rearrangement. During pupation myoblasts fuse with this scaffold to form the adult complement of sex-specific genital muscles. In general, however, development of the genitalia of this species is much more a product of rearrangement of larval tissue than in *Drosophila*;

in *Manducca* only the reproductive tract develops from genital discs (Thorn and Truman, 1989), while the genital discs form the reproductive tracts and the genitalia in *Drosophila* (see above). Development of the adult genital muscles around a larval template seems unlikely in *Drosophila* since the external and internal genitalia, attachment points for the genital muscles, form entirely from imaginal tissue.

In *Drosophila*, the DLMs and the use of larval templates for adult muscles, is the exception, not the rule. All other known adult muscles form solely from groups of cells set aside during embryogenesis (Bate, Rushton and Currie, 1991; Currie and Bate, 1991). How is the development of these muscles regulated? I will first review what is known about control of larval muscle development and then move on to control of adult muscle development.

Larval muscle pattern

Three possible mechanisms have been proposed for the regulation of development of larval muscles: 1) epidermal induction of the developing mesoderm, 2) autonomous control of pattern formation by the mesoderm, or 3) induction by the nervous system (Bate, 1990). In the developing embryo, the earliest observed primordial muscles are known as muscle precursors, fused doublets and triplets of cells. These precursors already occupy appropriate positions to later form the complete larval muscle pattern from their first appearance at eight hours after fertilization, which is prior to the outgrowth of axons from either sensory or motor neurons. Since the final pattern is observed prior to innervation, nerve activity or contact does not seem to play a role in

initial pattern formation. However, muscle precursors are first observed directly over the CNS and ectoderm, leaving open the possibility that patterning information could be transmitted from either of these tissues.

Epidermal differentiation precedes muscle differentiation, myoblasts segregate and fuse over an already differentiated cuticle (Bate, 1990). The cuticle could, therefore, provide patterning information to developing muscle. It is known that muscle will not form if the overlying cuticle is removed. Cuticle attachment points are required late in muscle differentiation, failure to form the proper pattern following cuticle removal may only show an inability of the muscle to differentiate, not a lack of determining information.

Hooper (1986) provides evidence implying that epidermis does not have a determining role. She showed that the homeotic gene *Ultrabithorax* (*Ubx*) has different apparent zones of action in the epidermis and muscle. *Ubx* transcripts and segments affected by Ubx mutations (areas showing ectopic expression of anterior structures) were offset by approximately 1.5 segments between the two tissue types. If this were a case of strict epidermal induction the same pattern of transformation in both tissues would be expected. While inductive communication across a gap of 1.5 segments is possible, its seems more likely that muscle development is not a function of direct induction by the epidermis.

With evidence against induction by either innervating nerves or the epidermis, mesoderm-autonomous patterning seems the most likely mechanism for patterning of larval muscle. However, recent work by Broadie and Bate (1993) on *Drosophila*

embryo synaptogenesis, formation of the connection between neuron and muscle cell, cautions against a strict autonomous versus induced model of pattern formation, but, instead promotes a more interactive model. They found distinct innervation-independent and innervation-dependent events in synaptogenesis. The muscle leads the motor neuron to the synaptic cleft (innervation-independent event) and subsequently the motor neuron directs the development of the receptive field (innervation independent events). While certain aspects of muscle pattern formation may be autonomous, formation of a normal neuromuscular junction, at least, requires interaction between muscle and neuron.

Adult muscle development

Following pupation adult flies emerge with a morphology vastly different from that of the larvae (Crossely, 1978). A distinct head, thorax and abdomen have developed, as have various appendages and the genitalia. The segments of the thorax contain a complex, but non-sex-specific pattern of muscles. In the abdomen the muscle pattern of the first six segments (A1 through A6) is also largely non-sex-specific. An important exception is the afore mentioned MOL (Lawrence, 1984). Additionally, the sexes differ in the muscles that surround the genitalia, the focus of this Masters Thesis.

Metamorphosis takes approximately 96 hours. As pupariation (formation of the pupa, which is different from the adult) begins the larval cuticle hardens and forms the pupal case. By 24 hours after puparium formation (APF) nearly all larval muscles

have been broken down and removed through histolysis and phagocytosis (Shatoury, 1956). Nerves also regress to a single major trunk in each hemisegment. With the exception of the previously mentioned DLM's (Shatoury, 1956; Fernades et.al., 1991) and the retained larval muscles (Crossley, 1978; Kimura and Truman, 1990) all adult cuticular muscles develop *de novo* during adult development (Bate et.al., 1991; Currie and Bate, 1991).

Adult muscles develop from mesodermal cells (Lawrence and Brower, 1982) set aside in the embryo (Bate et.al., 1991). In the thorax the muscle precursors are the adepithelial cells found associated with larval imaginal discs (Lawrence and Brower, 1982; Bate et.al., 1991). In abdominal segments A1 through A7 the muscle precursors are not associated with the precursors of the adult epithelium, but occur as four separate groups of cells; a ventral, a dorsal and two lateral groups (Bate et.al., 1991). The precursors of the genital muscles have not previously been described, in the third chapter I will present some preliminary evidence as to the location of these precursors within the genital disc.

Adult muscle precursor cells express the *twist* protein (Bate et.al., 1991), a protein initially expressed in all presumptive mesoderm (Thisse et.al., 1988), until midway in adult development. Decline in twist expression by these cells coincides with the fusion into myoblasts and the beginning of expression of muscle specific proteins (Currie and Bate 1991).

The *twist* protein has an interesting role in mesodermal development. It has primarily been of seen as interesting in regards to its role in embryogenesis, where it

is vital for gastrulation; *twist* flies fail to form any mesoderm and die at the end of embryogenesis (Beer et.al., 1987). While the function of the *twist* protein in embryogenesis has been relatively well studied (eg. Ip et.al., 1992), its function, if any, in the muscle precursor cells is unknown. The molecular characterization of the *twist* protein does suggest a possible mechanism for the action of the *twist* protein in the muscle precursor cells. The *twist* protein shows sequence homology with known basic helix-loop-helix (HLH) proteins (Murre et.al., 1989) and is localized to the nucleus (Thisse, 1988), where it acts as a transcriptional activator in presumptive mesoderm (Thisse et.al., 1988; Ip et.al., 1992). The HLH protein structure may indicate a possible DNA binding capability. In the muscle precursor cells the *twist* protein may act by binding to certain DNA regions and inactivating certain muscle-specific genes or activating certain genes responsible for repressing muscle cell development. At present this is all only interesting speculation.

During germ band retraction (mid-late embryogenesis) *twist* expression declines and becomes restricted to small, segmentally repeated, groups of 8-15 cells, the muscle precursor cells (Bate et.al., 1991). In developing pupae these cells proliferate, segregate and form the adult musculature (Curie and Bate, 1991).

During metamorphosis, groups of cells, called histoblasts, proliferate and spread out to form the new adult cuticle. Nerves grow out directly behind the developing epidermis. At this time the *twist* expressing muscle precursors also proliferate and spread out along the developing nerves (Currie and Bate, 1991). The muscle precursors migrate with the spreading histoblasts and nerve growth zones. By 24

hours APF (after puparium formation) the precursors are expressing muscle specific protein. By 28 hours they have begun to fuse, forming multinucleate cells. By 65 hours APF the final muscle pattern is established, cell fusion is complete and *twist* expression is gone (Currie and Bate, 1991).

Broadie and Bate (1991) used ablation experiments to show that, by the second instar, the *twist*-expressing cells form primordia for specific muscle subsets. Hydroxyurea (HU) is a DNA-synthesis inhibitor that blocks the activity of nucleotide reductase and kills cells as they pass through S-phase of the cell cycle. Adults developed from second instar larvae fed HU show ablation of groups of muscles. Ablations were found to show a quantal pattern, muscles were ablated as groups, not as single fibers. These groups are thought to correlate to the groups of twist-expressing muscle precursor cells.

How is the development and proliferation of these muscle precursor cells regulated to give rise to the pattern of muscles seen in the adult?

Adult muscle patterning

In the embryo growth of larval muscles follows complete epidermal formation and precedes outgrowth of nerve axons (Bate, 1991). In contrast, in the pupae, adult muscle develops concurrently with both the epidermis and nervous system (Bate et.al., 1991). In light of this important difference, patterning of adult and larval muscle may well be a function of different mechanisms. The three possible patterning mechanisms proposed by Bate (1990) for larval muscle; autonomous information,

epidermal induction or neural induction, have been investigated in patterning of adult muscle.

In the larval thorax the muscle primordia are associated with the imaginal discs (Lawrence, 1982). However, in the larval abdomen the primordia are not associated with the imaginal epidermis, the histoblast cells, but are closely associated with the peripheral nerves. The genital muscles may be an exception to this, as will be discussed in the third chapter. The association of the abdominal muscle precursors and the nervous system suggests that the nervous system may have a patterning role in the development of adult abdominal muscle; positioning or providing the information that positions the muscle precursors. Three facts argue against this possibility. First, segregation into the groups of adult-specific *twist* positive cells happens before outgrowth of the nervous system (Bate et.al., 1991); the initial pattern of *twist* positive cells cannot be a product of induction by the peripheral nervous system. Second, initial segregation of the cells is normal in *daughterless* (*da*) homozygotes although *da/da* flies do not form sensory neurons (Bate et.al., 1991). In *da/da* flies the final pattern of muscle cells is, however, distorted. This is interesting in light of, and generally consistent with, the Broadie and Bate (1993) paper on embryonic synaptogenesis; final pattern formation may be a function of an interaction between the nervous and muscle systems. Third, and most importantly, ablation of the innervating tissue does not prevent formation of the non-sex-specific muscle pattern (discussed below).

Similar to the earlier investigation of larval muscle patterning (i.e. Hooper, 1986), homeotic mutations have been used to investigate the role of the cuticle in patterning of the *twist* positive muscle precursors (Greig and Akam, 1993). It was found that the pattern of adult muscle precursors can be altered without necessarily altering the overlying cuticle, indicating that the muscle pattern does not require induction from the epidermis (Greig and Akam, 1993). In normal development the homeotic gene abdominal-A (*abd-A*) specifies the development of abdominal segments (Karch et al., 1990; Macias et al., 1990). Ectopic expression of *abd-A* in thoracic mesoderm of transformed larvae resulted in expression of an approximation of the abdominal pattern of *twist*-expressing cells (adult muscle precursors) in the thorax, without altering the thoracic cuticle. These results argue against mesodermal dependence on ectodermal induction for proper development. However, the converse experiment, ectopic expression of *abd-A* in the ectoderm, but not the mesoderm, was not reported. Without knowing the effect of ectopic *abd-A* expression in the ectoderm and considering that the transformed pattern of *twist* expressing cells was only an approximation of the normal pattern, it was not possible to rule out some role for the ectoderm in muscle patterning. Correct patterning of adult muscle may be a product of both autonomous and inductive signals.

Pattern formation in the fifth segment male muscle, the MOL

The mechanism determining the presence or absence of one particular muscle, the MOL, has been extensively studied (Lawrence and Johnston, 1984, 1986; Gailey

et.al., 1991; Taylor, 1992). The MOL is male specific; presumably its expression is in some way regulated by both the sex-determining genes and the muscle-determining genes. The developmental regulation of this muscle was originally investigated in terms of a "male" induction signal versus autonomous mesodermal patterning (Lawrence and Johnston, 1984;1985). Only more recently has the role of the specific sex-determining genes been investigated (Gailey et al., 1991, Taylor, 1992).

Lawrence and Johnston (1984) use gynandromorphs (XX flies with clones of X0 cells, XX//XO flies) to locate the region on the blastoderm where cells must be male (XO) for the male muscle to develop. This fate map, showing the relative location of the MOL patterning information in the developing blastoderm, was constructed by determining the probability that any two structures are of different genotype (phenotype) are on different sides of a clonal boundary. These probabilities are then used to create a two dimensional representation of the embryonic blastoderm showing the relative location of the primordia that give rise to adult structures. In general, the more often two structures are both in a clone, the closer their blastodermal primordia, and the closer their relative position in the map.

To create XO areas within an XX fly, lines of flies with specific mutations (*mitotic-loss-inducer, mit* and *paternal-loss-inducer, pal*) were used. These mutations cause loss of an X chromosome at a certain frequency, resulting in patches of X0 (male) tissue in predominantly XX (female) flies. These lines were constructed in such a way that X0 cuticle could be distinguished from XX cuticle by visible markers.

The presence or absence of a MOL was then scored against XX or X0 phenotypes in various tissues.

The focus for MOL patterning was found to map far away from the adult epidermis primordium and close to both the adult muscle and the nervous system primordia on the blastoderm fate map. In fact, the male patterning signal maps equidistant from both the adult muscle and nervous system primordia. Since the MOL focus does not map close to the primordium for the adult epidermis a patterning role for the epidermis is unlikely. But, because the patterning information maps equidistant from both the muscle and neural primordia it is not possible from these experiments to distinguish between these two regions as possible sources for patterning information.

These lines were constructed so that in a fraction of the flies loss of the paternal X chromosome leaves the clones *abd-B*$^-$, generating *abd-B*$^-$ clones in both the cuticle and the musculature. The *abd-B* mutation causes a transformation of A6 and A7 into A5. Lawrence and Johnston were, therefore, able to assay which tissue had to be male and *abd-B-* to give expression of a MOL in transformed tissue; could underlying cuticle induce the ectopic formation of a MOL, or was MOL formation dependent only on muscle genotype. As in the mapping experiment, the presence or absence of the MOL was found to be independent of cuticle genotype. However, because no independent muscle specific marker was included it was not possible from these experiments to determine whether the patterning signal was autonomous or came from innervating nervous tissue.

A subsequent set of experiments incorporated a marker capable of marking individual muscle cells (Lawrence and Johnston, 1986). Genetic mosaics were constructed by injecting heterozygous nuclei into host fly embryos, both host and donor tissue were assayed later for sex and genotype. Donor nuclei were homozygous for the dominant mutation *Miscadastral pigmentation* (*Mcp*). This mutation results in the transformation of A4 into A5, complete with ectopic expression of the MOL in males. Host flies carried a temperature-sensitive *succinate dehydrogenase (sdh)* allele, a mitochondrial marker. When host tissue was heated and then stained for SDH activity the tissue remains clear. In the injected flies only donor derived cells stain for SDH after heating. Cuticle-specific markers (*yellow* and *cinnabar*) were used to distinguish donor from host derived cuticle.

As in the gynandromorph experiments cuticle genotype was not found to influence MOL expression. Male muscles were seen associated with female cuticle in both A4 and A5. Similarly, male cuticle was not necessarily accompanied by the presence of a male muscle. Unexpectedly, in some cases XX muscle cells formed a male muscle in both A4 and A5 and in other cases XY tissue failed to form the male muscle. Further, expression of the male muscle in A4 (part of the *Mcp* phenotype) was independent of the state of the *Mcp* gene in the muscle or cuticle of that segment. Chromosomally male muscle was neither necessary, nor sufficient, for expression of the male phenotype. Apparently, formation of the MOL is neither controlled by cuticular induction nor cell-autonomous.

This leaves induction by the innervating tissue as the most likely candidate for the source of patterning information for the MOL. Note that the gynandromorph fate mapping experiment (Lawrence and Johnston, 1984) implicated autonomous or nervous induction as equally likely mechanisms; pattern induction by the innervating tissue was not a completely unexpected solution.

To a limited extent Lawrence and Johnston (1986) were able to examine the role of innervating tissue in determination of the MOL. The *sdh* marker labels motor neurons as well as muscle cells. Under the light microscope, however, stained terminals are only visible when they overlay unstained (sdh^+) muscle. In the cases where this condition was met the genotype of the innervating tissue did match the muscle pattern phenotype. Female innervating tissue corresponded to an absence of the MOL in both A4 (Mcp^- muscle) and A5. Further, in the two cases where male muscles formed in A4 from female, Mcp^+, tissue, the innervating tissue was both male and Mcp^-. These results implicate the innervating tissue as the focus of activity for both sex and segment specific patterning of the fifth segment MOL.

More direct evidence indicating a patterning role for the innervating tissue has come from subsequent ablation studies (Currie and Bate, in press). Denervation of a developing segment does not alter the pattern of non-sex-specific muscles. This is consistent with the model developed by Bate, patterning of the non-sex-specific abdominal muscles is not determined by induction by the nervous system. Control of the MOL is apparently different from the type of control found in other abdominal muscles.

Is this reliance on induction from innervating tissue a general feature of sex-specific muscle? In *Manducca* the larval muscle remnants form the normal sex-specific scaffolding even after denervation during muscle formation, but fail to recruit new myoblasts to regrow into adult muscles(Thorn and Truman, 1989). Regrowth, not patterning, is innervation dependent. However, this could be a consequence of development from larval templates and *Drosophila* genital muscles most probably do not form from a larval template. As yet there is no answer to this question, but preliminary studies of flies in which the terminal nerves are ablated during pupation shows that the genital muscles may depend on innervation for expression of some aspects of their normal sex-specific phenotype (Taylor, personal communication).

Summary

1) *Drosophila* are holometabolous insects, with different and distinct sets of larval and adult muscles.

2) In *Drosophila*, sex-determination is a function of the ratio of sex-chromosomes to autosomes. In most tissue this ratio is transmitted to sex-differentiation genes by a short cascade of sex-determining genes. In some tissues, most importantly the MOL, a modified cascade provides this function. The genes responsible for correct patterning of the genital muscles are unknown.

3) The pattern of larval muscles does not appear to be a result of epidermal or neural induction, but rather a result of autonomous patterning. The correct final pattern may, however, be the result of interaction between the tissues.

4) Most adult muscles develop *de novo* from precursor cells set aside in the embryo, not from larval muscles.

5) As in the larva, determination of adult non-sex-specific muscles is not a function of either epidermal or neural induction, rather it appears to be tissue autonomous.

6) Conversely, the determination of the fifth segment male muscle, the MOL, is non-autonomous and dependent on innervation by male (X0) tissue.

7) Whether determination of the sex-specific genital muscles is innervation-dependent or independent is unknown.

Chapter 2.

The Role of the *doublesex* Gene in the Determination of the Male and Female Genital Muscles of *Drosophila melanogaster*

Thomas J. S. Merritt

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Introduction

Adults of many species possess extensive sexual dimorphisms reflecting the different roles each sex plays in reproduction. Development of these sexually dimorphic structures and behavioral is a product of both tissue- and sex-specific regulation. Analysis of the development of sexually dimorphic characteristics in *Drosophila* has been aided by our extensive understanding of the genetic and molecular regulation of sexual determination (for recent review, Burtis, 1993). Genetic analysis has suggested that there are two output pathways that control the sexual differentiation of somatic tissues. In one pathway, the *doublesex* gene regulates peripheral development and at least one sexual difference in the central nervous system. In the second pathway, an unknown gene, or set of genes, regulates development of a male-specific muscle, the Muscle of Lawrence (MOL), and male sexual behavior (Taylor, 1992; Taylor, 1994).

Although numerous sexual dimorphisms have been described in adult *Drosophila*, the MOL is the only sex-specific muscle that has been extensively studied (Lawrence and Johnston, 1984; Lawrence and Johnston, 1986; Gailey et al., 1991; Taylor, 1992; Taylor and Knittel, in prep). A separate set of muscles, associated with the male and female genitalia, have previously been incompletely described (Ferris, 1950; Miller,

1950; Crossely, 1978). This study examines whether or not the development of these other sex-specific muscles, associated with the genitalia, are dependent on the *doublesex* branch of the sex-determination regulatory gene cascade.

In the development of sex-specific traits of many species, an initial chromosomal difference is translated into sexually dimorphic characteristics. In *Drosophila*, five genes translate the primary sex-determining signal, the ratio of X chromosomes to autosomes, into the signal for sexual differentiation: *Sexlethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*), *intersex* (*ix*) and *doublesex* (*dsx*) (for reviews: Baker and Ridge, 1980; Baker and Belote, 1983; Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Belote, 1992; Burtis and Wolfner, 1992; Cline, 1993).

Sxl, *tra* and *tra-2* act through the *dsx* gene to control proper female development. Mutations in *tra*, *tra-2* or the somatic tissue functions of *Sxl* cause females to develop as somatic males; these genes act to suppress masculinization in female development. Suppression of masculinization in XX flies also requires the product of the intersex (*ix*) gene. Somatic male development, on the other hand, is unaffected by the absence of function of these four genes.

Among these sex-determining genes *dsx* is unique in that a functional, sex-specific, *dsx* protein is required for proper sexual differentiation of either sex (Baker and Ridge, 1980; Baker and Wolfner, 1988; Burtis and Baker; 1989; Burtis et al., 1991). In females the *tra* and *tra-2* proteins work together to direct the female specific splice of the *dsx* primary transcript (Baker and Wolfner, 1988; Nagoshi et al., 1988; Burtis and Baker, 1989; Ryner and Baker, 1991; Hedley and Maniatis,

1991; Hoshijima et al., 1991). It has been proposed that the resulting female-specific protein, DSX^F, acts to repress male-specific development (Burtis and Baker 1980, Baker and Belote 1983) and activate female development (Taylor and Truman, 1992). In at least one case DSX^F is thought to directly control female-specific development by activating gene transcription (Burtis et al., 1991; Coshigano and Wensink, 1993). In males, a functional *tra* protein is absent and the *dsx* primary transcript is spliced into a male-specific pattern by default (Nagoshi et al., 1991). The resulting male-specific *dsx* protein, DSX^M, acts to repress female development (Burtis and Baker, 1980, Baker and Belote, 1983) and to activate male development (McRobert and Tompkins, 1985; Taylor and Truman, 1992; Jursnich and Burtis, 1993).

Absence of *dsx* function results in intersexual development of chromosomally male or female flies (Hildreth, 1965; Baker and Ridge, 1980; Postlethwait et al., 1980; Ota et al., 1981; Bownes and Nöthiger, 1981; Nöthiger et al. 1980, Chapman and Wolfner, 1988; Feng et al., 1991). In these intersexes, sex-specific structures, such as the genitalia, that develop from separate male and female primordia both develop. Structures, such as the analia, that develop from a single primordium differentiate as intermediate structures, exhibiting characteristics of both males and females. Other structures, such as the sex-combs, develop in *dsx* intersexes as an average of the male and female condition (Baker and Ridge, 1980).

Involvement on *dsx* in regulation of development of sex-specific traits is not, however, universal (McRobert and Tompkins, 1985; Taylor, 1992; Taylor et al., 1994). Although XX recessive homozygotes are intersexual in many traits, they do

not show any male courtship behavior. Similarly, XX; dsx^{D}/Df flies, which develop as somatic males, show no male courtship behavior. This suggests that the CNS is not completely transformed by *dsx* mutations (McRobert and Tompkins, 1985; Taylor et al., 1994). Additionally, formation of the MOL is unaffected by mutations in *dsx*; XX dsx^{-} intersexes do not express a MOL, while XY dsx^{-} intersexes always do (Taylor, 1992). With the number of sex-specific characteristics that develop independent of *dsx* regulation growing, I have examined the role of the *dsx* gene in the development of the only other known set of sex-specific muscles, the male- and female-specific genital muscles.

As a general rule, sex-specific structures develop in two ways: from the differentiation of a single primordium, which adopts a male or female morphology, or from the differentiation of one of a pair of dual primordia, each primordia with a fixed, sex-specific fate. In *Drosophila melanogaster*, development of structures from the genital disc involves both of these strategies. This imaginal disc is composed of three distinct primordia that give rise to the analia, male genitalia and female genitalia (Nöthiger et al., 1977; Schupbach et al., 1978; Epper, 1981; 1983a; Epper and Nöthiger, 1982; Epper and Bryant, 1982). A single anal primordia adopts either a male or female pattern, becoming either the two dorsal/ventral anal plates of the female or the two lateral anal plates of the male (Epper and Bryant, 1982; Taylor, 1989). By contrast there are two separate genital primordia within the disc: a female genital primordium that develops into the internal and external genitalia in females (Epper, 1983b; Taylor, 1989) and a male genital primordia that develops into the

internal and external genitalia in males (Epper and Bryant, 1982; Taylor, 1989). In a normal individual fly only one genital primordium differentiates; the other regresses and appears to degenerate (Epper and Bryant, 1982; Epper and Nöthiger, 1982; Epper, 1983; Taylor, 1989).

Materials and Methods

Drosophila stocks

The identification of genital muscles in wildtype flies was aided by the use of a P-element line (line 72-3, kindly supplied by Dr. S. Tobin), in which the expression of a reporter, ß-galactosidase, is driven by the promotor from a muscle-specific actin gene, 79B actin (*P[79B actin-lacZ, ry$^+$]*; Sanchez *et al.* 1983; Courchesne-Smith and Tobin, 1989).

I used both recessive and dominant mutant alleles of the *doublesex* gene to examine genital muscles in flies with intersexual development. For the purpose of mapping genital muscles in mutants, I constructed a *P[79B actin-lacZ, ry$^+$] p^p dsx^1 ry^{506} sr e^s/TM2, ry ubx^{130} e* line (*P[79B-lacZ] dsx^1/TM2*) by meiotic recombination and standard crosses. The *dsx$^-$* mutants examined in this study were either *P[79B-lacZ] dsx^1* homozygotes or *P[79B-lacZ] dsx^1/Df(3R)dsx15* transheterozygotes. The mutant transheterozygotes were generated by crosses between X/Y;*P[79B-lacZ] dsx^1/TM2* and *y w/y w;Df(3R)dsx15/ TM6B* flies and were used to control for potential background effects. Flies that were mutant for a dominant *dsx* allele and had the *P[79B-lacZ]*

insert were generated by crosses between either y^+ B^sY;dsx^D *Sb* e^s / *TM6B, Tb Hu* e^s *ca* (dsx^D/*TM6B*)or B^sY;dsx^M/*TM6B, Tb Hu* e^s *ca* (dsx^M/*TM6B*) males (provided by Drs B. S. Baker and R. Nagoshi) and females from the 72-3 line. Descriptions of the *dsx* and other marker alleles used are found in Lindsley and Zimm (1992).

Chromosomally male and female *dsx* intersexes are not distinguishable by external phenotype; chromosomal sex was, therefore, determined by three methods. The sex chromosome markers, *yellow (y)* and y^+Y, were used to distinguish *y*/y^+Y;*P[79B-lacZ] dsx1*/*P[79B-lacZ]* dsx^1 males (normal body color) from *y*/*y*;*P[79B-lacZ]* dsx^1/*P[79B-lacZ]* dsx^1 females (light body color). Likewise *y*/Y;*P[79B-lacZ]* dsx^1/*Df(3R)dsx15* males were distinguished from *y*/+;*P[79B-lacZ]* dsx^1/*Df(3R)dsx15* females. In some early experiments the *P[79B-lacZ]* dsx^1/*TM2* line had not been formed as a *y*/y^+Y line; in these cases XY *P[79B-lacZ]* dsx^1 homozygotes were sexed by the presence of the MOL muscle and XX dsx^1 by the absence of the MOL (Taylor, 1992).

All flies were raised at room temperature on a diet of sugar, cornmeal and agar, with propionic acid added as a mold inhibitor.

Visualization of genital muscles in wildtype and *dsx* mutant flies

In lines containing the *P[79B-lacZ]* insert, genital muscles were visualized by either monitoring ß-galactosidase activity or by immunohistochemical labeling of the enzyme. In either case, terminal abdominal segments from pharate (fully developed flies that have yet to emerge from the pupal case) or adult flies were dissected to

expose the genital muscles. For histochemical identification, abdomens were incubated in the X-gal reagent (0.2% 5-bromo-4-chloro-3-imdoxly-ß-D-galactopyranoside) dissolved in a reaction mixture (Asburner, 1989). Most abdomens were incubated in X-gal for 4 hours at room temperature. Longer incubation times did not change the pattern of stained genital muscles. After staining, abdominal segments were fixed for about an hour in 4% paraformaldehyde in 0.1M phosphate buffer (4% PFD in PBS) and mounted in Permount (Sigma) resin between two cover slips.

Abdominal preparations to be labeled for ß-galactosidase using immunohistochemistry were fixed in 4% PFD in PBS for 1 to 12 hours and then rinsed in PBS. Longer fixation times were found to reduce the level of background staining. Abdomens were initially blocked in 10% heat inactivated normal-goat-serum (NGS) in PBS for 1 hour and then incubated for 12 to 24 hours in anti-ß-galactosidase antisera (Cappel) at a dilution of 1:10,000 in 0.1M PBS containing 0.1% Triton-X and 2% heat-inactivated normal goat serum (PBS-TX-NGS). Following incubation in the primary antibody, the abdomens were incubated in a biotinilated secondary antibody (Vectakit, Vector Laboratory) at a dilution of 1:200 in PBS-TX-NGS. Finally, abdomens were incubated in the ABC reagent (Vector Laboratory). Diaminobenzidine (DAB; Sigma), in the presence of ß-D-glucose and glucose oxidase in 0.1 M Tris buffer (pH 8.2), was used as the chromogen (Metcalf, 1985). The preparations were mounted in Permount resin between two cover slips.

Terminal abdominal preparations were examined using polarized light or differential interference contrast optics to allow identification of both stained and non-stained muscle fibers. Muscle presence and staining were scored as separate phenotypes in both wildtype and the *dsx* mutant animals. Further, each side was scored as a separate case.

Results obtained by the use of either staining technique were consistent in both wildtype and *dsx* mutant flies. Antibody stained preparations were used to map the individual muscles and their insertion points in wildtype animals, since resolution of individual fibers and insertion points was better in these preparations. However, poor penetration of one or more of the immunohistochemical reagents often gave incomplete staining, especially in older animals. The analysis of muscle presence and staining included only wildtype and mutant animals stained for X-gal to insure high reproducability and sensitivity.

The pattern of stained muscles expands within 12 to 24 hours post eclosion to include a number of non-sex-specific muscle fibers in other regions of the abdomen. Segmental homologues of the MOL were found to stain in both males and females (Taylor and Knittel, in preparation). Additionally, ventral longitudinal and ventral transverse muscles stain in older animals. In animals stained as late as two weeks, no additional genital muscles were stained.

Results

In male and female *Drosophila* large, multi-fiber muscles attach to the genitalia and the terminal abdominal segments in a stereotypical, bilaterally symmetrical, pattern (Ferris, 1950; Miller, 1950; Figure 2.1A, Table 2.1A). In males and females from a *P[79B-lacZ]* line, a subset of these genital muscles expressed ß-galactosidase (Figure 2.1A and 2.1B, see Materials and Methods for details of the complete genotype). This subset of the genital muscles stained for reporter gene expression by midway through adult development, similar to the onset of staining in the MOL, another sex-specific muscle (Courchesne-Smith and Tobin, 1989; Taylor and Knittel, in prep). Early descriptions of abdominal muscles in *Drosophila* have only incompletely described the number and pattern of male and female muscles associated with the external genitalia (e.g. Ferris, 1950; Miller, 1950; Crossley, 1978); I have used the additional information obtained from the pattern of stained muscles in the *P[79B-lacZ]* line to further distinguish individual genital muscles and so to facilitate the comparison of wildtype muscles to those in *doublesex* mutants. In my analysis of genital muscles I have developed an identifying numbering system concerned only with the musculature having insertion points on the genitalia and the posterior abdominal segments. In cases where muscles had already been named or numbered, for example the penis extender muscle (Ferris, 1950), I include that information in

our description of the muscles. No muscles solely associated with the internal genitalia, such as muscles within the sperm pump or around the spermatatheca, are part of this study.

Figure 2.1 Composite photomicrographs of adult wildtype female terminalia. Muscles expressing the *P[79B-lacZ]* reporter gene were stained using an α-ß-galactosidase antibody (see Materials and Methods). Because of the thickness of the terminalia, at this magnification no single plane of focus shows all muscles and/or muscle attachment sites. The examples in this figure are, therefore, composites from images at multiple focal planes.

2.1A. Male internal terminalia. All 10 of the male genital muscles, mgm1-mgm10, are visible in this example. One of each bilateral pair is labeled. The structures of the male terminalia to which the mgms attach are: A6 tergite (6t); A6 ventral soft cuticle (6sc); genital arch (GA); penis apparatus (PA); penis apodeme (P); and the hypandrium (H).

2.1B. Female internal terminalia. Five of the female genital muscles are visible in this example, fgm4, fgm7, fgm8, fgm9 and fgm10. One of each bilateral pair is labeled. The structures of the female genitalia to which fgms attach are: A7 tergite (7t); A7 sternite (7s); A8 tergite (8t); and uterus (U). In this view, fgm1, fgm2, fgm3 and fgm5 are all hidden from view by the body of the uterus. fgm6, and its ventral insertion of the A7 tergite, are below the plane of focus and hidden by fgm7.

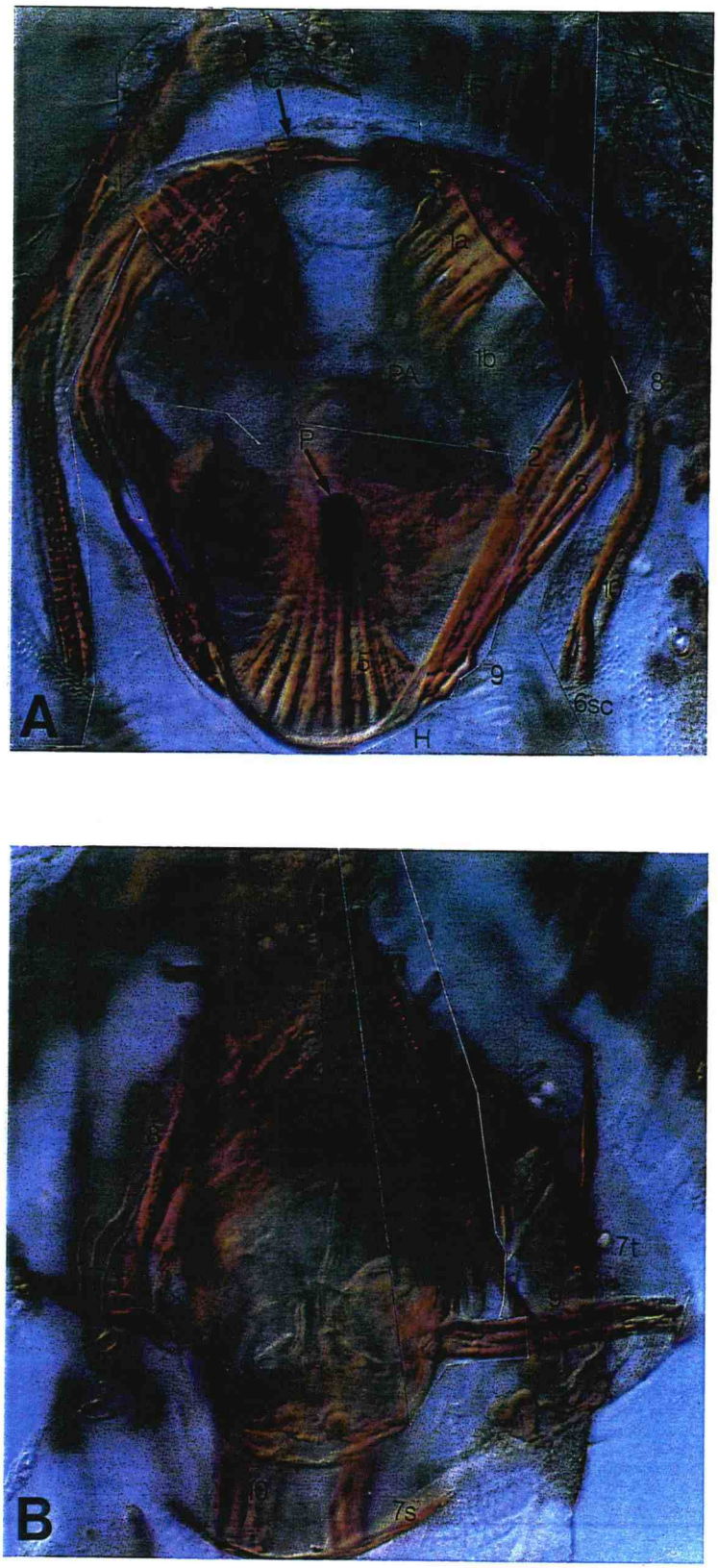


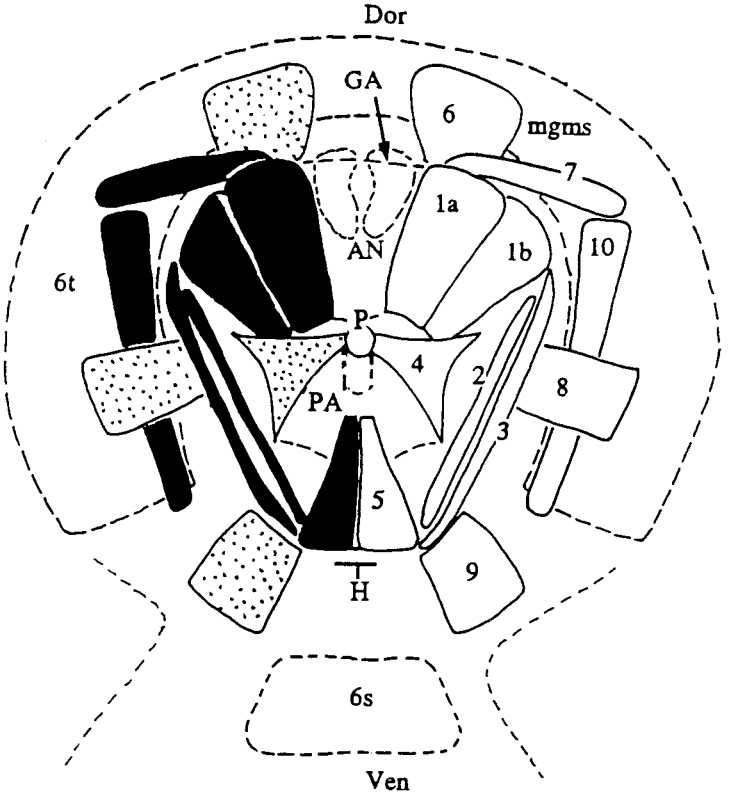
Figure 2.1

Figure 2.2 Schematic drawings of adult wildtype male and female terminalia. These drawings are adapted from camera lucida drawings and have been schematisized to more clearly demonstrate the relative positions of the genital muscles and their attachment points.

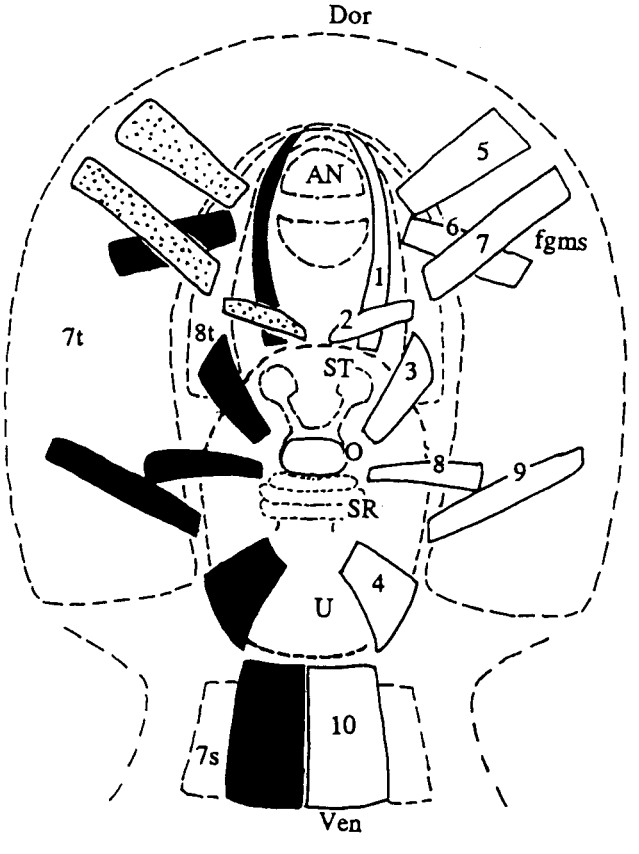
2.2A. Male internal terminalia. On the right, each of the male genital muscles is drawn in outline and numbered. On the left, muscles that stain for ß-galactosidase in flies carrying the *P[79B-lacZ]* reporter gene are uniformly blacked in; muscles that did not stain are stippled. The muscle attachment points, and other important structures within the male terminalia, are: A6 tergite (6t) and sternite (6s); genital arch (GA); anal plates (AN); penis apodeme (P); penis apparatus (PA); and the hypandrium (H). The anal plates lie posterior to (behind, in this drawing) the genital arch. All cuticular structures are drawn in a broken line.

2.2B. Female internal terminalia. As in the drawing of the male terminalia, on the right, each of the female genital muscles is drawn in outline and numbered. On the left, muscles that stain for ß-galactosidase in flies carrying the P[79B-lacZ] reporter gene are uniformly blacked in; muscles that did not stain are stippled. The muscle attachment points, and other important structures within the female terminalia, are: A7 tergite (7t) and sternite (7s); A8 tergite (8t); anal plates (AN); uterus (U); and two ducts that enter the uterus at the joint of the oviduct (O) with the uterus, the seminal receptacle (SR) and spermatathecae (ST).

In these drawings the anterior-posterior body axis is perpendicular to the plane of the paper, with anterior coming toward the reader. Dor is dorsal, Ven is ventral.


Dor
GA
6
mgms
7
1a
1b
10
AN
6t
P
4
2
8
PA
3
5
9
H
6s
Ven

A


Dor
AN
5
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fgms
1
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7t
8t
ST
3
O
8
9
SR
4
U
10
7s
Ven

B

Figure 2.2

Genital muscles in wildtype male flies

Associated with the external genitalia of wildtype males are ten multifiber muscles which I have called male genital muscles (mgms; Figure 2.1A). When visualized using polarized light, the number, position and size of these muscles was identical in Canton-S males and *P[79B-lacZ]* homozygous males (data not shown). Six of these muscles label for ß-galactosidase in the *P[79B-lacZ]* homozygotes (Figures 2.1A and 2.2A, Table 2.1A) and *P[79B-lacZ]* hemizygotes, which carry only one copy of the reporter gene construct (data not shown). Muscle staining for *P[79B-lacZ]* reporter activity was limited to skeletal muscles and was not found in muscles associated solely with the internal genitalia, such as the sperm pump and accessory glands.

For convenience, I have divided the ten male genital muscles into three sets, based on their anatomical location. The first set, mgm1-mgm5, includes the most central muscles - those which have both muscle attachment points on, or within, different structures of the external male genitalia. The most dorsal genital muscle, mgm1, attached to the anterior lip of the genital arch and onto the base of the penis apparatus. Two muscles, mgm2 and mgm3, attached laterally to the genital arch and extended ventrally to insert on the outer edge of the hypandrium. Although these muscles are similar, mgm2 can be distinguished by its slightly more dorsal insertion on the genital arch. The muscle recognized as the penis extender muscle by Ferris (1950), mgm4, attached to the hypandrium and the apex of the penis apodeme. The penis retractor (Ferris, 1950), mgm5, is the most ventral of the central set of male

genital muscles. It attached to the anterior (internal) ventral edge of the hypandrium and to the base of the penis apodeme.

Four of the five muscles from the central set stained for ß-galactosidase in *P[79B-lacZ]* males (Figures 2.1A and 2.2A, Table 2.1A). In all animals examined, the muscles mgm2, mgm3 and mgm5 stained uniformly and heavily, irrespective of whether one or two copies of the reporter gene were present. Within mgm1, muscle fibers in the more dorsal half of the muscle often stained more darkly than muscle fibers in the more ventral half. This differential staining of mgm1 was more common in pharate adults and adults with only a single copy of the *P[79B-lacZ]* reporter than in adults with two copies of the reporter gene. Under these conditions the X-gal product was often limited to the muscle nuclei and not distributed throughout the cytoplasm. Due to this differential staining, we denote the two regions of mgm1 separately as mgm1a and mgm1b. The only non-staining muscle in this central group was the penis extender muscle, mgm4; it was never found to stain for ß-galactosidase with either X-gal or anti-ß-galactosidase treatment.

Outside of this central ring were four muscles, mgm6-mgm9, which have one attachment point on the male genitalia and the other attachment point on the sixth (last) abdominal segment. Dorsally, mgm6 extended from the A6 tergite to the outside of the lip of the genital arch, opposite from the mgm1 insertion. The next muscle, mgm7, has one attachment point on the genital arch, in between mgm1 and mgm6, and a second attachment point on the A6 tergite. Laterally, mgm8 extended from the A6 tergite to the genital arch near the anterior insertion of mgm3.

Ventrally, mgm9 inserted on the lateral soft cuticle between the A6 tergite and sternite and extended to the anterior lip of the hypandrium, ventral to the insertion of mgm5. Of the muscles in this second set, only the dorsal muscle, mgm7, stained for ß-galactosidase.

The last male genital muscle, mgm10, is associated only with the terminal abdominal region. This muscle lies completely within A6, inserting dorsally on the tergite and ventrally on the soft cuticle between the tergite and sternite. This muscle has been included in our study, even though neither of its insertion points were on the genitalia, because it is male-specific and, like the genital muscles in the central and surrounding sets, stained for the *P[79B-lacZ]* reporter gene.

Genital muscles in wildtype female flies

A similar number of muscles have also been identified associated with the genitalia and terminal segments of wildtype females, the female genital muscles (fgms, Figures 2.1B and 2.2B). Of these ten muscles, six muscles stained for ß-galactosidase in females of the *P[79B-lacZ]* line. As in the male *P[79B-lacZ]* flies, muscular staining for ß-galactosidase was confined to skeletal muscles with attachments on the external genitalia and/or terminal segments; the circular muscles that surround the internal genital structures, such as the spermatatheca, uterus and seminal receptacle were never stained for reporter gene expression.

Similar to the male muscles, for convenience in description I divide the female muscles into two sets based on insertion points: a central and an external set. The

central set is composed of four muscles, fgm1 - fgm4, which have attachments to structures derived from the genital disc. Three of the muscles, fgm1, fgm2 and fgm3, attached to the A8 tergite and the uterus. The most dorsal muscle, fgm1 (called muscle *148* in Miller, 1950), inserted dorsally, near the midline, on the A8 tergite and attached ventrally to the base of the uterus. More laterally, fgm2 (muscle *146*; Miller, 1950) extended from the ventral part of the A8 tergite to the base of the uterus at the midline, crossing internally over fgm1. Another lateral muscle, fgm3 (muscle *147*; Miller, 1950) attached to the ventral edge of the A8 tergite and extended up, in a basket-like pattern, around the dorsal side of the uterus, ending about two thirds of the way along the uterus. The most ventral of the central set of muscles, fgm4, (muscle *149*; Miller, 1950), extended from the base of the uterus up, along the ventral side of the uterus, to insert at the same level as the attachment of fgm3 to the uterus. In the central set of female genital muscles, fgm1, fgm3 and fgm4, but not fgm2, stained for ß-galactosidase (Figures 2.1B and 2.2B).

The external set of female genital muscles contains six muscles, fgm5-fgm10, which surround the female genitalia (Figures 2.1B and 2.2B). In the dorso-lateral region, three muscles attached to both the A7 and A8 tergite have previously been identified as a single muscle (muscle *139*; Miller (1950). The most dorsal of the three, fgm5, extends along the anterior-posterior body axis between A7 and A8. From a more lateral position, fgm6 extends obliquely between the tergites, inserting on A8 in the same region as fgm5. The third muscle in this group, fgm7, inserts on the A7 tergite between fgm5 and fgm6 and extends, like fgm5, along the anterior-

posterior body axis, crossing fgm6, to insert more ventrally on the A8 tergite. Of these dorso-lateral muscles only fgm6 stained for ß-galactosidase in *P[79B-lacZ]* females (Figures 2.1B and 2.2B).

The last three muscles of this external group each attach to the uterus. From a lateral attachment in the A7 tergite, fgm8 extended to the body of the uterus, inserting at the same level as the epidermal ducts of spermatatheca and seminal receptacle. This is the longest of the female muscles and, along with fgm1, the easiest to identify in wildtype females. Near to fgm8, fgm9 extended from the A7 tergite to the base of the uterus. Along the ventral midline, fgm10 (*144*; Miller, 1950) stretched from the anterior edge of the A7 sternite to the ventral region at the base of the uterus. All three of these muscles, fgm8, fgm9 and fgm10, stained for ß-galactosidase in *P[79B-lacZ]* females(Figures 2.1B and 2.2B).

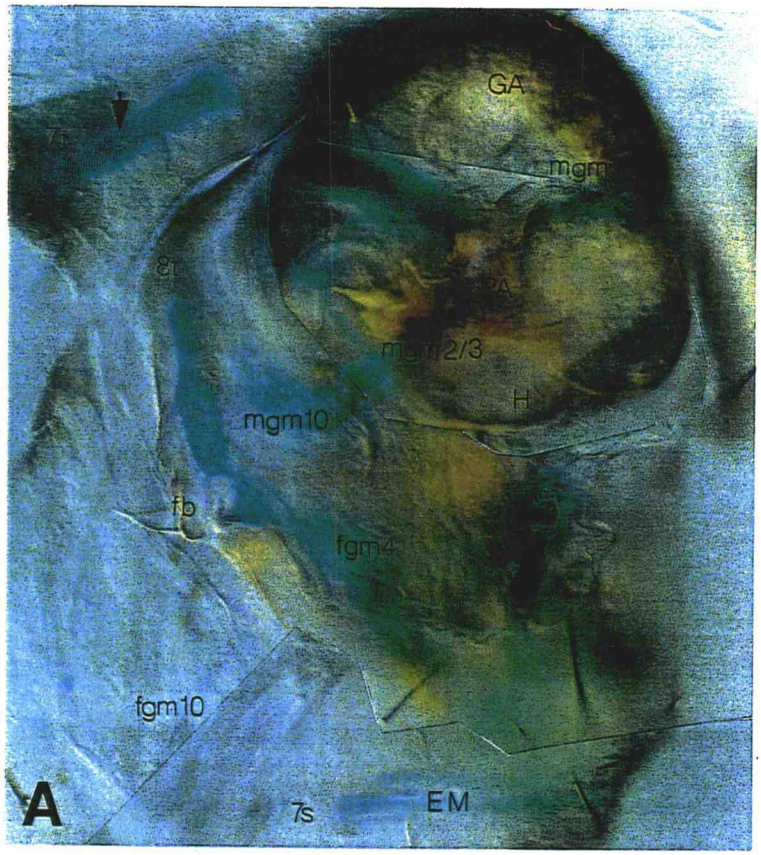
Figure 2.3 Composite photomicrographs of adult, *dsx*-mutant, intersexual terminalia. Muscles expressing the P[79B-lacZ] reporter gene were stained using the X-gal reagent (see Material and Methods). As in Figure 1, these are composite images created from photomicrographs at multiple planes of focus.

2.3A XY; dsx^1/dsx^1 intersexual terminalia. One from each pair of genital muscles present is labeled. The structures of the terminalia to which genital muscles attach are: A7 tergite (7t); A7 sternite (7s); A8 tergite (8t); genital arch (GA); penis apparatus (PA); hypandrium (H); soft cuticle of the female genitalia (in this image, a female bristle, fb, marks one edge of this region). In this example, both of the pair of mgm1s are present and show *P[79B-lacZ]* activity. A pair of mgm2/3 are also present, but only the left (our view) muscle shows *P[79B-lacZ]* activity. Only one mgm10 is present; it also shows *P[79B-lacZ]* activity. No other male genital muscle is present in this example; notice the lack of muscle fibers between the hypandrium and the penis apparatus and between the genital arch and the terminal tergite. A pair of fgm4 are found at the mouth of the genital knob; both show *P[79B-lacZ]* expression. fgm10 is present, but shows no expression of the *P[79B-lacZ]* reporter gene. A thin example of the ectopic staining muscle fibers (EM) is present between the right A7 tergite and sternite.

In some animals, stray muscles, that could not be identified as male or female genital muscles, were present in the terminalia. The stained muscle in the dorsal right half of the A7 tergite is an example of such a muscle (marked with arrow). It is possible that this particular muscle represents an fgm6 with a posterior insertion in A7, instead of A8 (no muscle extends between A7 and A8 on this side of the animal) but we have no way of verifying that identification. Such unidentifiable muscles were uncommon.

2.3B XX; dsx^1/dsx^1 intersexual terminalia. Muscles and muscle attachment points are labeled as in 3A. In this example 1 mgm1 is present. It stains for *P[79B-lacZ]* expression. Similarly, 1 mgm2/3 is present and stained. Two mgm10 are present and show *P[79B-lacZ]* activity. No other male genital muscles are present in this example. Again, notice the lack of muscle fibers between the hypandrium and penis apparatus, between the hypandrium and the soft cuticle ventral to the hypandrium or between the genital arch and the terminal tergite. fgm4 is present and stained for *P[79B-lacZ]* expression in both sides of this animal. On the right side one fgm5/7 is present. Though out of this plane of focus, fgm10 is present, but unstained; it lies anterior, and internal (toward the reader) of the genital knob. No other female genital muscle is present in this example. Notice the lack of staining fibers between the A7 tergite (the bristles on the right side of this example are on the A7 tergite) and sternite (out of focus in this view). In addition, in this example, a spermatethecae (ST), one of the ducts that empties into the uterus, is present over the mouth of the genital knob (GN). The staining in the genital knob, not associated with any muscle fibers, is non-specific; similar staining is seen in wildtype uteri containing eggs.

2.3C XX;*dsx*^{M}/+ intersexual terminalia. Muscles and muscle attachment points are labeled as in 3A. In this example, mgm1, mgm2/3 and mgm10 is each present and stains for *P[79B-lacZ]* activity on both sides of the animal. Small, unstained, muscles extend between the genital arch and the A8 tergite (marked with arrow). These could be rudimentary examples of mgm6 or mgm8. No other male genital muscles are present is this example. One fgm6 is present, and shows *P[79B-lacZ]* activity, on the left side of the animal; no fgm6 was present on the other side of the animal (out of this view). fgm 10 is present on both sides, but unstained. No other female muscles are present. Note the presence of the large ectopic staining muscle (EM) spanning between the A7 tergite and sternite on both sides.


GA
7t
mgm
8t
PA
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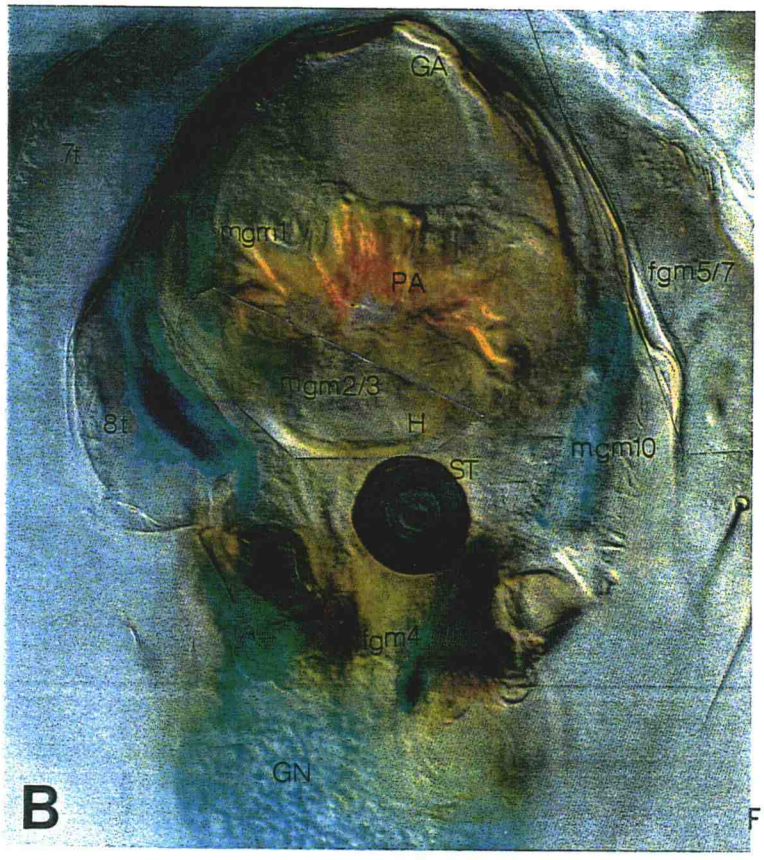
H
mgm10
fb
fgm4
fgm10
A
7s
EM


GA
7t
mgm1
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8t
H
mgm10
ST
fgm4
GN
B

Figure 2.3

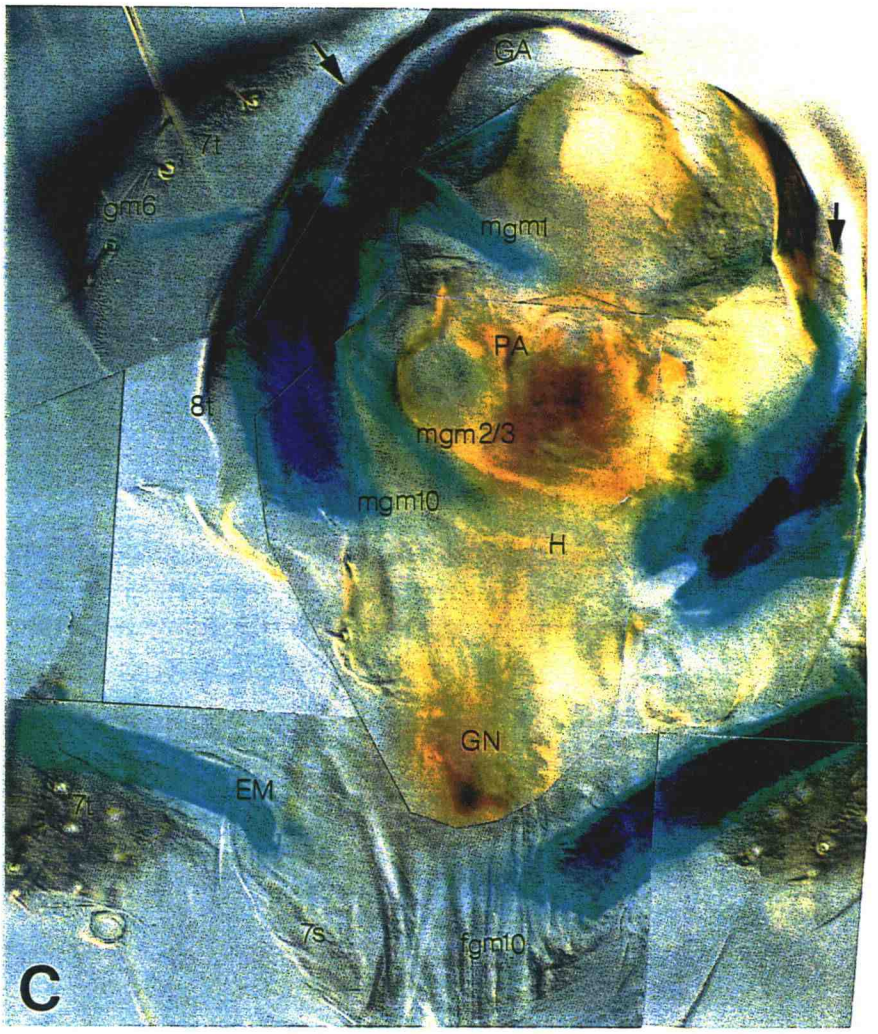


Figure 2.3

Figure 2.4 Schematic drawing of the adult *dsx* mutant terminalia.

The pattern of genital muscles was similar in both the XX and XY *dsx*-recessive and XX *dsx*-dominant intersexes (see Table 2.1) and is represented here in a single composite drawing. The largest difference in the terminal muscles between the dominant and recessive mutants was in the frequency and size of the ectopic staining muscle (EM; see Results)

On the right, each of the genital muscles is drawn in outline and numbered. On the left, muscles that stain for ß-galactosidase in mutant flies carrying the *P[79B-lacZ]* reporter gene are uniformly blacked in; muscles that did not stain are stippled. The muscle attachment points, and other important structures within the intersexual terminalia, are: A6 tergite (6t) and sternite (6s); A7 tergite (7t) and sternite (7s); A8 tergite (8t); genital arch (GA); anal plates (AN); penis apodeme (P); penis apparatus (PA); and the hypandrium (H). In the majority of intersexual flies examined, a uterus was not identifiable. Instead the female genitalia were everted, to a greater or lesser degree depending on the individual, from the terminalia in cuticular pouch, the genital knob (GN). The anal plates lie posterior to (behind, in this drawing) the genital arch; the genital knob is external, posterior to the A7 sternite. All cuticular structures are drawn in a broken line. In these drawings the anterior-posterior body axis is perpendicular to the plane of the paper, with anterior coming toward the reader. Dor is dorsal, Ven is ventral.

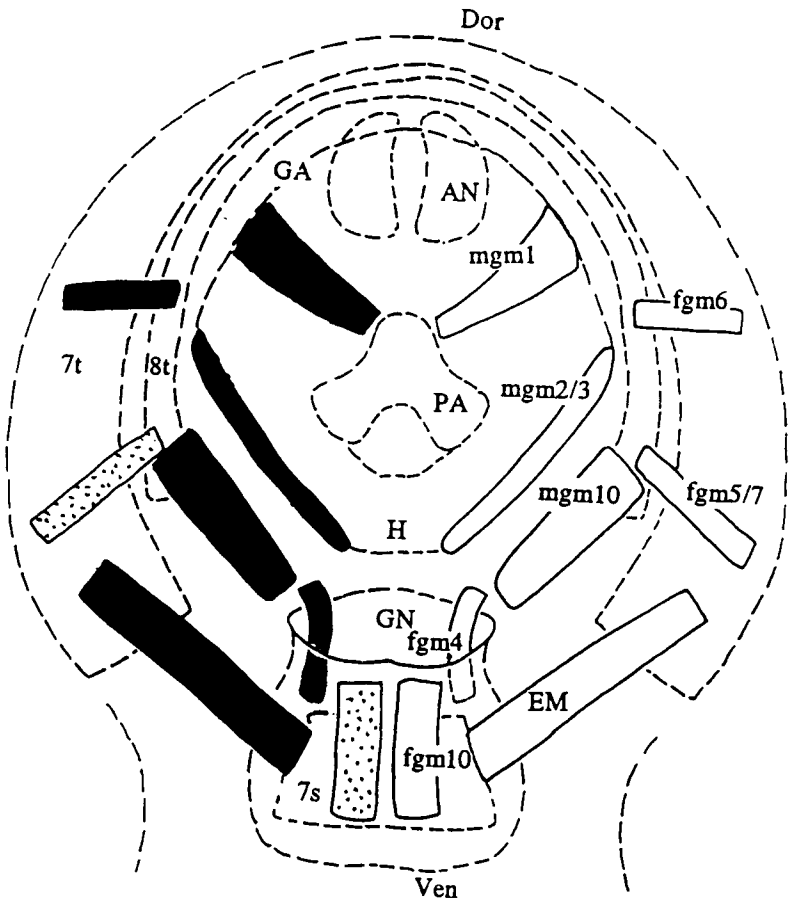

Dor
GA
AN
mgm1
fgm6
7t
8t
PA
mgm2/3
mgm10
fgm5/7
H
GN
fgm4
EM
fgm10
7s
Ven

Figure 2.4

A subset of the muscles associated with the genitalia of wildtype flies is found in *dsx* mutant intersexes

To determine the role of *dsx* in the development of sex-specific genital muscles, I identified the male and female muscles that developed in animals mutant for recessive and dominant alleles of *dsx*. Chromosomally male and female *dsx*$^-$ mutants develop both male and female genitalia, becoming nearly identical intersexes (Hildreth, 1965; Baker and Ridge, 1980). I foresaw three possible ways in which male and female genital muscles might be arranged in these mutants: 1) two complete sets of muscles, one set associated with the male genitalia and one associated with the female genitalia; 2) only one set of muscles, which would be distributed in some fashion between male and female genital structures; or 3) no muscles might develop at all. I examined the genital muscles in two recessive *dsx*$^-$ genotypes, *P[79B-lacZ] dsx*^{1}/*P[79B-lacZ] dsx*^{1} and *P[79B-lacZ] dsx*^{1}/*Df(3R)dsx15*. The results obtained from XX and XY *dsx*$^-$ mutants were the same (Table 2.1A and 2.1B) and will be included within a single description.

Male genital muscles in *dsx*$^-$ intersexes

In both XX and XY *dsx*$^-$ intersexes a largely complete, but distorted, set of external male genitalia differentiate (Hildreth, 1965; Baker and Ridge, 1980). Among the genital structures clearly identifiable are the genital arch, lateral plates and claspers. The penis apparatus, and accompanying hypandrium are present, but reduced; the penis apodeme is especially poorly developed (Hildreth, 1965, Baker and Ridge, 1980).

Only a fraction of the normal male genital muscles are present in the *dsx*⁻ intersexes (Figure 2.1 A and 2.2A and table 2.1A). All distinctly identifiable male muscles belong to the group of six male genital muscles that stained for ß-galactosidase in the *P[79B-lacZ]* line. Because the pattern of stained muscle in wildtype flies having either one or two copies of the *P[79B-lacZ]* reporter gene was consistent, I had expected the presence of stained muscles to provide positive identification of a subset of the genital muscles in *P[79B-lacZ] dsx*^{1} homozygotes and *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* mutant transheterozygotes. Unexpectedly and in contrast to the uniformity of staining between wildtype individuals, not all muscles that were identified, by their attachment points, as ß-galactosidase-positive muscles stained for ß-galactosidase in the *dsx*⁻ mutants. Some muscles consistency found to stain in wildtype flies occasionally failed to stain in *dsx*⁻ mutants. I have, therefore, treated muscle presence and muscle staining as two separate phenotypes (Table 2.1A and 2.1B). The frequency of muscle staining was very similar between *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* flies, flies with only a single copy of the *P[79B-lacZ]* insert, and *P[79B-lacZ] dsx*^{1}*/P[79B-lacZ] dsx*^{1} flies, flies with two copies of the insert (Table 2.1A). It was unlikely, therefore, that our inability to detect ß-galactosidase-expressing fibers was a function of the number of reporter genes present.

Of the male muscles clearly identified in *dsx*⁻ mutants, three, mgm1, mgm2 and mgm3, were in the central group of muscles, which have both attachment points within the genitalia. In close to 93% of the cases examined from either recessive genotype was found (Table 2.1A). In the *dsx*⁻ mutants, mgm1 was often smaller than

in wildtype flies and in over a third of the cases in which mgm1 was present that muscle was unstained. The difference in staining separating mgm1a from mgm1b was not identifiable because of the variability in muscle staining; subsequently it was not possible to distinguish between the these two regions in *dsx*$^-$ mutants (Table 2.1A). Muscle fibers extending from the genital arch to the hypandrium, in positions appropriate to either mgm2 or mgm3, were found in almost all of the *P[79B-lacZ] dsx*^{1} homozygotes and *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* mutant transheterozygotes (Table 2.1A). As was the case for mgm1, these muscles appear proportionally smaller than in wildtype male genitalia and it was generally not possible to distinguish two separate muscles, or to distinguish the more dorsal insertion of mgm2. I have, therefore, scored only for the presence or absence of muscle fibers in this region, referring to any muscle fibers present as mgm2/3. These mgm2/3 fibers stained for ß-galactosidase in only about one half of the *dsx*$^-$ cases (Table 2.1A).

The only other identifiable male genital muscle found in these intersexes was mgm10. In wildtype males, mgm10 spans the most terminal abdominal segment, A6, extending ventrally from the tergite to the sternite. In contrast to wildtype males, XX and XY *dsx*$^-$ intersexes have a pronounced A7 segment and A8 tergite, similar to the segmental pattern found in wildtype females (Baker and Ridge, 1980). Though much smaller than the A6 tergite, the A8 tergite is interposed between the dorsal male genital structures and the A7 tergite and thus has the position similar to the A6 tergite in wildtype males, wrapping around the genital arch and lateral plates. In 69% of the *dsx*^{1} homozygotes and 93% of the *dsx*^{1}*/Df(3R)dsx15* transheterozygotes (Table 2.1A) a

muscle was attached dorsally to the A8 tergite and ventrally to a point below the hypandrium; this muscle appears to correspond to mgm10 of wildtype males. No muscle with attachment points in A6, that would correspond to mgm10, was found. In *dsx*⁻ intersexes, mgm10 was always found to stain (Table 2.1A).

Qualitatively there appears to be a correlation between overall muscle size and staining in mutant animals. mgm10, the most regularly stained of the male genital muscles, was also the largest genital muscle in these mutants. In contrast, the unstained mgm1 and mgm2/3 were often smaller than their staining counterparts (data not shown). This is not to say, however, that a large size was a prerequisite for staining; some single fiber muscles were found to stain. Instead staining seems to follow some degree of muscle development, with which size is often correlated.

Two of the genital muscles that label for ß-galactosidase in wildtype male *P[79B-lacZ]* flies, mgm5 and mgm7, were never detected in *dsx*⁻ mutants. This is unlikely to be simply the lack of proper insertion points on the cuticle; the areas onto which these muscles would be expected to attach were recognizable and other identified muscles, such as mgm1, attached near by. In wildtype males, mgm5 extended from the ventral lip of the hypandrium to the base of the penis apodeme. The lip of the hypandrium was particularly well formed in these *dsx*⁻ intersexes and, while the penis apparatus is reduced, it too can be identified. Similarly, in wildtype males, mgm7 extended from the genital arch to the A6 tergite, however, no muscle was found in this region of *dsx* mutant intersexes, although the genital arch was well formed.

Further, the insertion points for mgm7 were near those of two other muscles, mgm1 and mgm10; both muscles which were found in the *dsx*⁻ intersexes.

Because of the complexity and distortion of the genitalia in *dsx*⁻ intersexes, identification of the four male genital muscles that do not stain in *P[79B-lacZ]* males, mgm4, mgm6, mgm8 or mgm9, was more difficult. In wildtype males the penis extender, mgm4, attached to the penis apodeme and the hypandrium (Figures 2.1A, 2.2A). In contrast, no muscle fibers were ever found in this region of the *dsx*⁻ intersexes, though a hypandrium and penis apparatus were both recognizable in all cases examined. In wildtype males, mgm9 also attached to the hypandrium, but no similarly located muscles were found in the *dsx*⁻ mutants. The presence of the female genitalia ventral to the hypandrium *dsx*⁻ mutants makes it difficult to predict where mgm9 would attach to the terminal abdominal segment, but the attachment of any muscle to the hypandrium would be recognizable.

Two other muscles, mgm6 and mgm8, that attached to the external genitalia and the last abdominal segment, were not found in the *dsx*⁻ mutants. In some cases small dorsal groups of fibers were found to extend between the genital arch and the A8 tergite, but these were proportionally much smaller than any mgm6 found in wildtype males. The mgm8 muscle would be expected to attach near the middle of mgm2/3 and span between the genital arch and the A8 tergite. No muscles were found to attach to the lateral plate and the A8 tergite in this area. If mgm6 or mgm8 were present at all, in either the *P[79B-lacZ] dsx*^{1} homozygotes or the *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* mutant transheterozygotes, it was only in a greatly reduced form.

Occasionally, stray muscle fibers were present, irregularly attached to the male genitalia in the *dsx*⁻ intersexes, but these did not attach at points that would be expected from mgm6, mgm8 or any other genital muscle. Besides these small muscle fibers no other ectopic, or unusual, muscle fibers were associated with the male genitalia in *dsx*⁻ intersexes.

In summary, about half of the normal number of male genital muscles were detected in XX and XY *dsx*⁻ intersexes. Two of the male genital muscles, mgm1 and mgm10, were clearly present at a high frequency in the *dsx*⁻ intersexes. Lateral genital muscles were also represented, although it could not be determined whether mgm2, mgm3 or both muscles were present. While staining of these muscles for ß-galactosidase in mutants with one (*P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* flies) or two (*P[79B-lacZ] dsx*^{1} homozygotes) of the *P[79B-lacZ]* construct was not as consistent as seen in wildtype flies it was sufficient to positively identify these four muscles. Four male genital muscles, mgm4, mgm5, mgm7 and mgm9 were never detected in *dsx*⁻ intersexes even though at least one attachment point could unambiguously be identified. Finally, two of the male genital muscles, mgm6 and mgm8, did not appear to be present, although it could not be completely ruled out that infrequently appearing fibers represented these two muscle groups.

Female genital muscles in *dsx*⁻ intersexes

Along with the fairly well-developed set of male genitalia, XX and XY *P[79B-lacZ] dsx*⁻ intersexes also possess a less well-developed set of female external and

internal genitalia (Hildreth, 1965; Baker and Ridge, 1980). In 6% of the *P[79B-lacZ] dsx*^{1} homozygotes we examined, and 12% of the *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* flies, an identifiable uterus and seminal receptacle were present. In most of the remaining *dsx*$^{-}$ flies no uterus was identifiable, instead a mass of cuticle and internal tissue had been everted from the region of the female genitalia, forming a protrusion referred to by Hildreth (1965) as a genital knob. This knob often contained one or more spermatethecae. Spermatetheca were also often present within the body cavity, both in flies with and without uteri. One *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* fly expressed both a genital knob and a very reduced uterus and seminal receptacle (data not shown).

Two female genital muscles, fgm4 and fgm10, were readily identified with the female genitalia. In wildtype females, fgm4 inserts posteriorly on the base of the uterus and anteriorly about two thirds of the way along the body of the uterus (Figures 2.1B and 2.2B). In approximately 85% of *dsx*$^{-}$ intersexes in which a uterus was found, a muscle was found to extend anteriorly from the base of the uterus to the body of the uterus, with attachment points appropriate for fgm4. In those cases in which no obvious uterus formed, the internal female genital structures were generally partially everted into a protruding knob. A muscle often extended from the mouth of the knob, into the cavity. Based on its attachment points I have identified this muscle as an everted fgm4. Overall, 93% of the *P[79B-lacZ] dsx*^{1} cases and 86% of the *P[79B-lacZ] dsx*^{1}*/Df(3R)dsx15* cases expressed a fgm4 (Table 2.1B). This muscle was found to stain in most cases examined from either *dsx*$^{-}$ genotype (Table 2.1B). The only other clearly identifiable female muscle found with the female genitalia of *dsx*$^{-}$

mutants was fgm10 (Table 2.1B). As in wildtype females, this muscle inserted on the A7 sternite and on the base of the uterus or, in those cases in which no uterus was found, on the cuticle ventral to the genital knob. This muscle was variable in its size, ranging from as large as in wildtype females to as small as a quarter the number of fibers with a noticeable disruption of their regular, ordered pattern. Further, fgm10 was never found to stain for ß-galactosidase in pharate, or recently emerged, adult *dsx*$^{-}$ intersexes. fgm10 did stain in some older adult cases, but only in flies old enough for the non-sex-specific dorsal and ventral muscles to also stain for ß-galactosidase. By contrast, in wildtype females fgm10 always had early, sex-specific expression of ß-galactosidase in the *P[79B-lacZ]* line.

Due to the distortion of the terminalia, the reduction of the female genitalia and the lack of internal female landmarks in the *dsx*$^{-}$ intersexes it was more difficult to identify some individual female genital muscles. In the majority of cases examined, muscles corresponding to fgm5, fgm6 or fgm7, were found dorsally, spanning between the A7 and A8 tergites. However, in *dsx*$^{-}$ intersexes three separate muscles were rarely found in this region; on average fewer than 2 muscles were present (1.6 +/- 0.8 in *P[79B-lacZ] dsx*^{1}*/P[79B-lacZ] dsx*^{1}, 1.8 +/- 1.0 in *P[79B-lacZ] dsx*^{1}*/Dfdsx15*). In a small percentage (less than 12%) of *dsx*$^{-}$ cases, a stained muscle, which would correspond to fgm6, the only dorsolateral muscle to stain for ß-galactosidase in wildtype flies, was present. In those cases where no stained muscles was present the unstained muscles could have corresponded to either fgm5, an unstained fgm6 or fgm7. On the basis of the presence of cases with a stained muscle,

I have inferred that fgm6 was present, but unstained in most *dsx*⁻ intersexes. In reporting the data in Table 2.1B I have made the assumption that fgm6 was always present, if any muscle was found spanning the A7 and A8 tergites. At least one muscle, putatively fgm6, was present in approximately 93% of the *P[79B-lacZ] dsx*^{1}*/P[79B-lacZ] dsx*^{1} cases and 81% of the *P[79B-lacZ] dsx*^{1}*/Dfdsx15* cases, but stained in less than 12% of those cases. In most of the cases in which a stained fgm6 was present unstained fibers were also present. The presence of unstained fibers suggests that either fgm5 and/or fgm7 were also present in the *dsx*⁻ intersexes. It was only possible to distinguish fgm5 from fgm7 when they were separated by a stained fgm6. This was rarely the case. Any muscles in this region, other than fgm6, were, therefore, scored as fgm5/7 (Table 2.1B).

Five of the ten female genital muscles were not detected in the *dsx*⁻ intersexes: fgm1, fgm2, fgm3, fgm8 and fgm9. None of the dorsal three muscles in the central group, fgm1, fgm2 or fgm3, the muscles attaching to both the A8 tergite and the uterus or genital knob, were ever identified in these intersexes, even in the 6% to 12% of cases where a uterus was present. Because, in *dsx*⁻ intersexes the anal plates are surrounded by the genital arch it is difficult to predict what form fgm1 would take in these flies, but its dorsal insertion site, the A8 tergite, was always identifiable and its ventral insertion site, the dorsal edge of the uterus, was often identifiable, but no appropriate muscle was ever detected. The other central muscles, fgm2 and fgm3, insert on the A8 tergite and the uterus in wildtype females, but no such muscles were detected in *dsx*⁻ intersexes. No muscles were found to insert on both the A7 tergite

and the uterus or genital knob, as would be expected of either fgm8 or fgm9. Of the six muscles with attachment points on the A7 or A8 tergite and the uterus in wildtype females only fgm4 was found in the *dsx*$^{-}$ intersexes.

In summary, in addition to the three or four male genital muscles, four or five of the female genital muscles found in wildtype females were found in *dsx*$^{-}$ intersexes: fgm4, fgm10, fgm6 and either, or both, fgm5 and fgm7. Staining of these muscles for ß-galactosidase was not as consistent as in *P[79B-lacZ]* females. Five of the female genital muscles were never found: fgm1, fgm2, fgm3, fgm8 and fgm9.

2.1A. Muscles associated with the male genitalia.

GENOTYPE		N	Percentage of sides with muscle			
			mgm1	mgm2/3[a]	mgm5,mgm7	mgm10
null alleles						
P[79B-lacZ] dsx^{1}/P[79B-lacZ] dsx^{1}	XX	30	97(45)	98(45)	0(0)	83(100)
P[79B-lacZ] dsx^{1}/P[79B-lacZ] dsx^{1}	XY	36	100(74)	90(40)	0(0)	93(100)
Df(3R)dsx15/P[79B-lacZ] dsx^{1}	XX	32	95(39)	87(89)	0(0)	69(100)
Df(3R)dsx15/P[79B-lacZ] dsx^{1}	XY	18	93(82)	79(55)	0(0)	93(100)
dominant alleles						
dsx^{M}/P[79B-lacZ] dsx^{+}	XX	40	94(63)	93(51)	0(0)	100(100)
dsx^{D}/P[79B-lacZ] dsx^{+}	XX	48	94(28)	84(42)	0(0)	96(100)
dsx^{M}/P[79B-lacZ] dsx^{+}	XY	10	100(100)	100(100)	100(100)	100(100)
dsx^{D}/P[79B-lacZ] dsx^{+}	XY	10	100(100)	100(100)	100(100)	100(100)
control genotypes						
P[79B-lacZ]/P[79B-lacZ]	XY	30	100(100)	100(100)	100(100)	100(100)
dsx^{M}/P[79B-lacZ] dsx^{1}	XX	5	100(100)	100(100)	100(100)	100(100)
dsx^{D}/P[79B-lacZ] dsx^{1}	XX	5	100(100)	100(100)	100(100)	100(100)

Table 2.1A. Muscles associated with the male genitalia.

GENOTYPE		N	Percentage of sides with muscle			
			mgm1	mgm2/3[a]	mgm5,mgm7	mgm10
null alleles						
P[79B-lacZ] dsx^1/P[79B-lacZ] dsx^1	XX	30	97(45)	98(45)	0(0)	83(100)
P[79B-lacZ] dsx^1/P[79B-lacZ] dsx^1	XY	36	100(74)	90(40)	0(0)	93(100)
Df(3R)dsx15/P[79B-lacZ] dsx^1	XX	32	95(39)	87(89)	0(0)	69(100)
Df(3R)dsx15/P[79B-lacZ] dsx^1	XY	18	93(82)	79(55)	0(0)	93(100)
dominant alleles						
dsx^M/P[79B-lacZ] dsx$^+$	XX	40	94(63)	93(51)	0(0)	100(100)
dsx^D/P[79B-lacZ] dsx$^+$	XX	48	94(28)	84(42)	0(0)	96(100)
dsx^M/P[79B-lacZ] dsx$^+$	XY	10	100(100)	100(100)	100(100)	100(100)
dsx^D/P[79B-lacZ] dsx$^+$	XY	10	100(100)	100(100)	100(100)	100(100)
control genotypes						
P[79B-lacZ]/P[79B-lacZ]	XY	30	100(100)	100(100)	100(100)	100(100)
dsx^M/P[79B-lacZ] dsx^1	XX	5	100(100)	100(100)	100(100)	100(100)
dsx^D/P[79B-lacZ] dsx^1	XX	5	100(100)	100(100)	100(100)	100(100)

Table 2.1B. Muscles associated with the female genitalia.

GENOTYPE		N	Percentage of sides with muscle						
			fgm1	fgm3	fgm4	fgm6[b]	fgm5/7[a,b]	fgm8, fgm9	fgm10
null alleles									
P[79B-lacZ] dsx[1]/P[79B-lacZ] dsx[1]	XX	30	0(0)	0(0)	93(97)	92(0)	53(0)	0(0)	100(0)
P[79B-lacZ] dsx[1]/P[79B-lacZ] dsx[1]	XY	36	0(0)	0(0)	94(89)	94(6)	56(0)	0(0)	100(0)
Df(3R)dsx15/P[79B-lacZ] dsx[1]	XX	32	0(0)	0(0)	93(80)	87(8)	63(0)	0(0)	100(0)
Df(3R)dsx15/P[79B-lacZ] dsx[1]	XY	18	0(0)	0(0)	78(71)	76(12)	68(0)	0(0)	100(0)
dominant alleles									
dsx[M]/P[79B-lacZ]	XX	40	0(0)	0(0)	65(90)	91(6)	74(0)	0(0)	100(0)
dsx[D]/P[79B-lacZ]	XX	48	0(0)	0(0)	89(82)	84(15)	82(0)	0(0)	100(0)
control genotype									
P[79B-lacZ]/P[79B-lacZ]	XX	30	100 (100)	100 (100)	100 (100)	100 (100)	100 (0)	100 (0)	100 (100)

Genital muscles in flies expressing dominant *dsx* alleles

Dominant *dsx* alleles transform XX flies into intersexes, while XY flies develop as normal males (Baker and Ridge, 1980; Table 2.1A). Wildtype XY flies express a male-specific *dsx* transcript that is translated into a male-specific protein, DSXM. Similarly, wildtype XX flies express a female-specific *dsx* transcript that is translated into a female specific *dsx* protein, DSX^F. XX flies with a single copy of a dominant *dsx* allele and a copy of a dsx^+ allele generate both the male- and female-specific transcripts (Nagoshi and Baker, 1990) and so should produce both DSX^M and DSX^F proteins. Recent work on a small number of phenotypes has suggested that *dsx* plays a positive role in sex-specific development; the presence of DSX^M or DSX^F being required to activate development of some sex-specific characteristics (Taylor and Truman, 1992; Coshigano and Wensink, 1993; Jursnich and Burtis, 1993). If the presence of DSX^M or DSX^F was required for normal development of any of the genital muscles I would expect *dsx*-dominant mutants to have muscles not seen in recessive *dsx* mutants, or to produce larger or more frequent examples of muscles already present in the null cases. I have examined the effects of two dominant *dsx* alleles, *dsx-Dominant* (dsx^D) and *dsx-Masculinizer* (dsx^M), on the genital musculature (Figure 2.3C; Table 2.1).

Male genital muscles in dsx^{Dom} intersexes

The same subset of mgms found in recessive mutant intersexes, mgm1, mgm2/3 and mgm10, was present in the XX;*dsx*-dominant intersexes. The frequency of

occurrence of these muscles was also similar to that seen in the recessive genotypes (Table 2.1A). For example, mgm1 was present in 93-100% of *dsx*$^-$ animals and 94-100% of the XX;*dsx*^{DOM}/*P[78B-lacZ] dsx*$^+$ animals. In these XX; *dsx*-dominant intersexes, mgm2 and mgm3 were not distinct muscles, having the same appearance as in the *dsx*$^-$ intersexes. Again, the frequency with which mgm2/3 formed was similar in XX;*dsx*^{DOM}/*P[78B-lacZ] dsx*$^+$ animals was similar to that with which it formed in XX and XY *dsx*$^-$ intersexes. In the XX;*dsx*^{DOM}/*P[78B-lacZ] dsx*$^+$ flies mgm10 was found in A8, as it was in the *dsx*$^-$ intersexes. Further, the six mgms not found in the recessive *dsx* intersexes, mgm5 and mgm7, were likewise missing from the XX;*dsx*-dominant intersexes (Table 2.1A).

As in the *dsx*$^-$ intersexes, muscles that could be identified by their attachment points as muscles that expressed the *P[79B-lacZ]* reporter in wildtype flies did not always stain in XX;*dsx*^{DOM}/*P[78B-lacZ] dsx*$^+$ animals. mgm10 stained in essentially all cases of either dominant genotype. Approximately one half of the mgm2/3's were found to stain. The fraction of mgm2/3 and mgm10 that stained in XX;*dsx*-dominant animals was similar to that found in the recessive *dsx* mutants (Table 2.1A). mgm1 stained in almost two thirds of the XX;*dsx*^{M}/*P[79B-lacZ]* cases examined and just less than a third of the XX *dsx*^{D}/*P[79B-lacZ]* cases. It is worth noting that, in general, XX *dsx*^{M}/*P[79B-lacZ]* intersexes show a more male-like phenotype than XX *dsx*^{D}/*P[79B-lacZ]* intersexes (i.e. better developed external male genitalia and more poorly developed female genitalia, Baker and Ridge, 1980). The higher frequency of

staining of male genital muscles in XX *dsx*^{M}*/P[79B-lacZ]* intersexes than in XX *dsx*^{D}*/P[79B-lacZ]* intersexes may be a reflection of this.

XX flies that carry a dominant *dsx* allele over a *dsx* recessive allele or a *dsx* deficiency (for example: *dsx*^{D}*/dsx*^{1} or *dsx*^{M}*/dsx*^{1}) develop as somatic males, pseudomales (Baker and Ridge, 1980). The XX; *dsx*^{M}*/P[79B-lacZ] dsx*^{1} and XX; *dsx*^{D}*/P[79B-lacZ] dsx*^{1} pseudomales expressed a set of genital muscles identical in number, morphology and staining to that found in XY *P[79B-lacZ]* homozygotes. The normal complement of male muscles was also present in *dsx*^{D}*/Df(3R)dsx15* flies, though these flies lacked the insert and could not be scored for ß-galactosidase staining. No fgm was ever detected in the *dsx*-dominant/*dsx*$^{-}$ pseudomales.

Female genital muscles in dsx^{Dom} intersexes

The muscles found associated with the female genitalia were also very similar in the dominant and recessive mutant intersexes (Table 2.1B). fgm4 was found in the majority of the cases examined and stained in over 70% of those cases in which it was found. fgm10 was found in all cases, but was never stained in pharate and recently eclosed flies. As with the *dsx*$^{-}$ intersexes, I have made the assumption that fgm6 was always expressed in cases where muscles were present between the A7 and A8 tergites. fgm6 was found to stain in 6% of the *dsx*^{M} intersexes and 12% of the *dsx*^{D} intersexes. Additional muscles in this dorsal region were identified as fgm5/7; these were found in 74% of the *dsx*^{M} cases and 82% of the *dsx*^{D} cases. No other fgm could be detected in the *dsx*^{Dom} intersexes, though, as in the *dsx*$^{-}$ intersexes, the

insertion points for fgm1, fgm2, fgm3, fgm8 and fgm9 could be identified.

Ectopic muscles in *dsx*-dominant intersexes

The majority of the genital muscles expressed in the *dsx*$^-$ and dominant *dsx* intersexes were the same, however, one unusual muscle was found much more often in dominant than in recessive *dsx* mutants, a possible case of activation by DSX^M. A muscle that extended from the A7 tergite to the A7 sternite was found in less than 15% of XX and XY *dsx*$^-$ intersexes (Figure 2.3A). A similar muscle was present in 72% of XX;*dsx*^{D}/*P[79B-lacZ]* cases and 85% of the XX;*dsx*^{M}/*P[79B-lacZ]* cases (Figure 2.3C). In general, this muscle was much larger in *dsx*^{Dom} animals than in *dsx*$^-$ animals. When present in either *dsx*^{Dom} or *dsx*$^-$ animals this muscle stained for ß-galactosidase in pharate adults. Thin muscle fibers do extend between the tergite and sternite of every segment in both male and female wildtype flies, but these muscles have been found to stain only in flies older than 24 hours post-eclosion. It is important to point out that this staining muscle was in A7, entirely separate from the genitalia, this was not a genital muscle, but an masculinization of a segment not found in wildtype males.

Discussion

Muscles associated with the male and female genitalia and terminal segments

I have described two sets of sex-specific muscles that attached to the genitalia and terminal abdominal segments of adult flies (Figures 2.1A, 2.1B, 2.2A, 2.2B). A subset of both male and female muscles expressed a reporter gene construct driven by the 79B actin gene promotor (*P[79B-lacZ]*) by mid-pupation. Among abdominal muscles only the MOL also shows this early pattern of reporter gene expression (unpublished observations; Taylor and Knittel, in prep; Currie and Bate, in press). Sex-specific expression of the *P[79B-lacZ]* reporter was, then, confined to morphologically distinct, sex-specific, muscles in the abdomen. Interestingly, I find that both males and females had a similar number of genital muscles, with a similar fraction of those muscles showing *P[79B-lacZ]* activity. These similarities in muscles and staining pattern are in contrast to the dramatically different external and internal male and female genitalia that develop from discrete and separate primordia within the genital disc (Nöthiger et al., 1977; Schupbach et al., 1978; Epper, 1981; Epper and Nöthiger, 1982; Epper, 1983a; Epper and Bryant, 1983). Given the difference in external and internal genitalia, there was no reason, *a priori*, to expect this degree of similarity between the male and female genital muscles. In fact, given the difference in potential insertion points, it seemed more likely that the genitalia would have had notably different numbers of attaching muscles.

With the external differences in the genitalia there was a noticeable morphological similarity between some of the male and female genital muscles. The best example of this similarity was between fgm5, fgm6 and fgm7, linking A7 and A8 in females, and mgm6, mgm7 and mgm8, linking A6 and the genital arch in males. These are dorsal muscles, all from the set of external muscles that surround the genitalia in either sex. In either group, only the central muscle stains for reporter gene activity in the *P[79B-lacZ]* line. The outer two muscles do not stain and, to a large extent, resemble slightly modified versions of the non-sex-specific longitudinal muscles found in every abdominal segment. Based on their similar morphologies and patterns of staining for reporter gene activity these six muscles appear to be segmental homologues. The dorsal most male and female muscles, mgm1 and fgm1, are also similar, and possibly homologous, both inserting dorsally to the genitalia, anterior to the anal plates, and ventrally to parts of the internal genitalia. The similar number, pattern of staining for a muscle specific reporter gene and, at least in some cases, morphology, suggests that, unlike the rest of the external and internal genitalia, the male and female genital muscles may develop from a single primordium.

Where might this muscle primordium be? The muscles in the adult abdominal segments develop from imaginal cells associated with the abdominal segmental nerves in the larvae (Bate et al., 1991; Currie and Bate; 1991). The genital muscles precursors could be similarly associated with the terminal nerve. However, development of the genitalia is more like development of thoracic structures, such as the wing, than it is like development of the rest of the abdomens, since both the wing

and the genitalia develop from imaginal discs. Thoracic muscle precursors are located within the thoracic imaginal discs (Lawrence, 1982; Bate, et al., 1991). The genital muscle precursors might, similarly, be located within the genital disc. In fact, when male and female genital discs were labeled with an antibody specific for muscle precursor cells, a large number of cells, associated with both the male and female genital primordia, do show specific staining (see chapter 3). While many of these cells probably give rise to the visceral muscles associated with the internal genitalia, the location of other cells over areas of the disc epithelium that will develop into external structures make those cells strong candidates for the genital muscle precursors.

The development of the genital muscles is dependent of *dsx* activity

A primary finding of this study was that *dsx* does have a role in the development of sex-specific muscles. Previous work had showed that the MOL develops independent of *dsx* control (Taylor, 1992), leading us to wonder if all sex-specific abdominal muscles develop independent of *dsx*. We find that in *dsx* loss-of-function mutations, which result in the development of both male and female genital structures, many of the appropriate genital muscles also form (Figures 2.3A, 2.3B; Table 2.1). Further, in concert with finding similar cuticular structures, in XX and XY *dsx* null mutants muscle development is also similar in pattern and reporter expression. While it was not possible, *a priori*, to predict what effect, if any, *dsx* would have on expression of these muscles, only if *dsx* did have a role in regulating their

development would XX and XY *dsx* mutants express similar genital muscles. I conclude, therefore, that *dsx* does have a role in the development of these genital muscles and that *dsx*-independence is not a feature common to muscle development.

The only phenotype, other than the MOL, that has been shown to develop independently of *dsx* activity is male courtship behavior (McRobert and Thompkins, 1985; Taylor et al., 1994). Courtship behavior is presumably controlled by the CNS. Interestingly, development of the MOL is also controlled by innervating tissue (Lawrence and Johnston, 1984; 1986; Currie and Bate, in press). *dsx*-independence and some role of the CNS may be linked, but this possibility should be taken cautiously as expression of at least one sex-specific CNS phenotype does require *dsx* activity (Taylor and Truman, 1992). It is not yet known whether innervation has any role in development of the genital muscles, but in light of the dependence on proper *dsx* activity, it is interesting to speculate that it will be less than in the MOL. I started this project asking whether *dsx*-independence was a general feature of sex-specific muscle development, instead, *dsx*-independence or dependence may be more a function of the role of the CNS in the development of a phenotype.

A subset of male and female genital muscles form in *dsx*$^-$ intersexes

To understand how the *dsx* gene was involved in the control of the development of the male and female genital muscles I examined the effect of *dsx* loss-of-function alleles on the pattern of adult genital muscles. I postulated three possible ways in which muscles might be distributed between the male and female genitalia found in

dsx⁻ intersexes: 1) two complete sets of muscles; 2) one set of muscles, distributed between the male and female genitalia; or, 3)no muscles at all. I can rule out the third possibility immediately, distinction between the first two requires closer examination. If both sets of muscle had developed then 20 muscles would have been expected, compared to about ten muscles if only one set had developed. Based on insertion points and reporter gene staining characteristics, between 3 and 6 male genital muscles and 4 or 5 female genital muscles were present in any of the *dsx*⁻ intersex cases we examined, strongly suggesting that only the equivalent of one set of genital muscles formed in the mutants (Figures 2.3A, 2.3B). In contrast to the muscles in wildtype flies, the individual genital muscles in these intersexes were not consistently present or stained (Table 2.1). The regularity of formation and frequency of staining appeared to be a characteristic of the individual muscles, not dependent on the degree of development of particular mutant flies. For example, mgm1 was found in over 90% of all cases examined, whereas fgm5/fgm7 was found in between 50% and 80% of the cases examined. Other muscles were never detected; for example mgm7 in males and fgm3 in females.

The failure of certain muscles to form could result from either the absence of the necessary muscle insertion points, or too few muscle precursor cells. I favor the second possibility. Any distortion in the pattern of muscles present could be a result of a distortion in the cuticle to which the muscle attaches. However, in many cases in which the cuticle appears to be a suitable substrate for muscle attachment no muscle forms. For example, although mgm7 was never found in the *dsx* intersexes,

its prospective insertion points on the genital arch and terminal tergite were both identifiable, and nearby points were used by other muscles. While I cannot rule out some defect in the cuticle, making it an unsuitable attachment site, as the cause of the failure of certain muscles to form, the nearby insertion of other muscles makes this a less likely possibility.

The similarity in number and pattern of genital muscles in wildtype flies (Figures 2.1A, 2.1B, 2.2A, 2.2B) already suggests that these muscles develop from a single group of precursors. If the reduction in the number of male and female genital muscles expressed in the *dsx* intersexes was a result of a limited number of available myoblasts I would expect the total number of muscles in the intersexes to be similar to that found in the wildtype flies. I find distinct examples of only two of the mgms, mgm1 and mgm10. Muscles were also present in the intersexes, that could have corresponded to either, or both, mgm2 and mgm3. Three muscles, mgm4, mgm5 and mgm7, were never found in the intersexes. The remaining male genital muscles, mgm6, mgm8 and mgm9, were never clearly identified, either they were not present, or were expressed in a very reduced form that was not easily detected. In total, I find three or four distinct male genital muscles, depending on whether both mgm2 and mgm3 were present, with the remote possibility of 3 rudimentary muscles. Three of the female genital muscles were identified in the dsx^{-} and dsx^{Dom}/+ intersexes, fgm 4, fgm6 and fgm10. Other muscles in the intersexes appear to correspond to either fgm 5 or fgm7 or both. In all I find 4 or 5 of the female genital muscles. Summing the male and female muscles gives a minimum estimate of 7 genital muscles in the *dsx*

intersexes, and including both possible muscles from groups that I cannot distinguish raises this number to 9, including the male muscles possibly expressed in a reduced form raises the maximum number of muscles to 12, out of a possible 20 genital muscles. This is consistent a reduction in the number of muscles present due to a limiting number of myoblasts.

It may seem problematic that certain muscles always fail to develop while others form in almost all cases; limited resources might be expected to lead to random loss, not regular loss, of individual muscles. Why do certain muscles appear to develop at the expense of others? It is possible that certain of the genital muscles preferentially receive myoblasts, at the expense of nearby muscles, resulting in the consistent appearance of some muscles and absence of others in this case of limited resources. This could reflect a developmental bias, with earlier developing muscles reducing the number of myoblast available for later developing muscles or could result from certain muscles being able to actively recruit myoblasts at the expense of others. A developmental bias does seem to be involved in control of development of the MOL and the non-sex-specific longitudinal muscles that surround it (Taylor and Knittel, in prep). When developing muscles were challenged by artificially limiting the number of myoblasts available, the MOL was found to always do better than the adjacent muscles at acquiring myoblasts. In counting the number of nuclei in the resulting MOL and surrounding longitudinal muscles, it was found that by some means the MOL always received a greater percentage of myoblasts than other nearby muscles (Taylor and Knittle, in prep).

Along with a reduction in the total number of muscles associated with the genitalia I were surprised to find that muscle presence was not always accompanied by muscle staining (Table 2.1). This lack of staining could simply have been a result of too few nuclei expressing the *P[79B-lacZ]* product for us to detect. In many cases, however, single fibers, with a small number of nuclei, stained using both X-gal and the anti-ß-galactosidase antibody. Further, in other cases, large muscles, left to stain for long periods of time (up to 24 hrs), failed to show any staining. It seems unlikely, then, that the lack of staining simply results from an insufficient number of muscle nuclei. Instead, if we regard staining as a separate phenotype from muscle expression, lack of staining may result from an inability of the muscle to completely differentiate into its sex-specific phenotype. This implies that at least one aspect of muscle phenotype, *P[79B-lacZ]* activity and therefore presumably 79B-actin expression, is more sensitive to *dsx* activity that other aspects,such as muscle position and growth, of the muscle.

A possible positive role for DSX^M

In most sex-specific phenotypes that have been studied the male or female *dsx* proteins act as negative regulators, turning off the expression of inappropriate sexual characteristics. The presence of the male *dsx* protein, or the female *dsx* protein, has been shown to be required for the development (not repression of development) of a few sexually dimorphic phenotypes. For example, the yolk proteins, normally produced only in females, require the presence of DSX^F for normal levels of expression (Coshigano and Wensink, 1993). Similarly, the male-specific division

pattern of a set of abdominal neuroblasts requires the presence of DSX^M (Taylor and Truman, 1992). In either of these cases removal of the *dsx* gene activity causes a loss of the phenotype which can be restored genetically by the return of the appropriate *dsx* protein.

It is possible that the missing genital muscles, or the reduction in frequency of staining in the muscles identified, resulted from the lack of DSX^M, or DSX^F, in flies expressing *dsx* loss-of-function alleles. To test whether the presence of either *dsx* protein was required for development of any of the genital muscles I examined XX; *P[79B-lacZ]* dsx^{DOM}/+ flies. If the normal development of any of the genital muscles required either DSX^M or DSX^F I would expect to see an increase in the number of male muscles present, or possibly an increase in the frequency of staining compared to either XX or XY *dsx*$^-$ intersexes. However, I find no significant increase in the total number of identifiable genital muscles present, the frequency with which any muscle was found, nor the frequency with which a male muscle stained for the reporter, between the dominant and recessive intersexes (Table 2.1).

There is, however, one muscle that is found in approximately 65% more *dsx*-dominant cases than *dsx*-recessive cases. This was the large, staining muscle extending between the A7 tergite and A7 sternite in over 70 percent of the *dsx*-dominant intersexes examined (Figure 2.3a, 2.3C). This muscle was present in less than 15% of the *dsx*-intersexes and was generally smaller than when found in the *dsx*-dominant intersexes. This muscle appears to be ectopic since no such stained muscle

is found in wildtype male or female flies, though a band of small, non-staining muscle fibers does extend from tergite to sternite in every abdominal segment of both sexes.

The position of this muscle was strikingly similar to that of mgm10, the lateral staining muscle in A6 of wildtype males and A8 of both *dsx*-dominant and *dsx*$^-$ intersexes. A7 is not present in wildtype males, but is in females and *dsx* mutant intersexes. I interpret this A7 muscle as the ectopic expression of a male phenotype in these *dsx*-dominant intersexes. While expression of mgm10 itself does not require DSX^M expression (see above), the presence of DSX^M appears to transform a non-sex-specific muscle into a male phenotype. Ectopic expression of DSX^M has been shown to result in expression of sex-comb-like bristles on all of the legs (Jursnich and Burtis, 1993); this may be a similar ectopic activation of a male phenotype.

Chapter 3.

Identification of Putative Genital Muscle Precursor Cells in Wildtype and *dsx*-Dominant Genital Discs

Thomas J. S. Merritt

Introduction

During pupation the largely non-sex-specific Drosophila larvae metamorphose into sexually dimorphic adults. Metamorphosis involves drastic changes throughout the body of the developing fly, including changes in the epidermis, the nervous system and the musculature. This overall change is accomplished through both rearrangement of larval tissues and creation of adult structures *de novo* from imaginal cells. In the last chapter I described a set of sex-specific muscles associated with the adult genitalia, here I address a possible source of those muscles.

In general, the muscles present in adult insects develop either through a rearrangement of larval muscles and subsequent fusion of myoblasts, or through development of the muscles entirely from cells set aside earlier (reviewed in Nüesh, 1985; Bate, 1993). In the only previously studied case of development of insect genital muscle, the genital muscles of *Manducca sexta* were shown to develop around a patterning template of larval muscle remnants (Thorn and Truman, 1989). However, this type of muscle development is rare in Drosophila; adult muscles generally develop entirely from cells set aside in the embryo (Lawrence, 1982; Lawrence and Brower, 1982; Bate et al., 1991). The only adult muscle in Drosophila whose development is known to involve a larval template is a set of thoracic indirect

flight muscles, these muscles form around a scaffolding of partially histolysed larval muscle remnants (Fernandes et al., 1991). Recently, however, the development of these adult muscles has been demonstrated to be independent of the presence of these larval muscle remnants, at least at the level of gross muscle patterning (Fernandes, personal communication).

The muscle precursors, myoblasts, of adult muscles in Drosophila express the *twist* protein (Bate et.al., 1991), a protein which is initially expressed in all presumptive mesoderm (Thisse et.al., 1988), until midway in adult development. A decline in *twist* expression by these cells coincides with the fusion of myoblasts into myotubes, followed by muscle differentiation, which can be measured by the onset of transcription of muscle-specific genes (Currie and Bate, 1991). For the flight and leg muscles in the thorax, muscle precursors are found associated with larval imaginal discs (Lawrence, 1982; Bate et.al., 1991). In abdominal segments A1 through A7 the muscle precursors are not associated with the precursors of the adult epithelium, but occur as four separate groups of cells located at a ventral, a dorsal and two lateral sites (Bate et.al., 1991). While adepithelial cells, presumed myoblasts, have been located in the genital disc, their relationship to particular genital muscles has not been described.

The genitalia, like thoracic structures, develop from an imaginal disc, the genital disc. Gynandromorph and somatic recombination studies (Nöthiger et al., 1977; Schupbach et al., 1978; Epper and Nöthiger, 1982) as well as metamorphosis of disc fragments (Epper, 1981; Epper, 1983a; Epper and Bryant, 1983), have shown that the

single imaginal disc contains three separate primordia: a male primordium, a female primordium and an anal primordium. All three primordia are identifiable in discs from third instar larvae of either sex. Sex specific cell growth and movement during metamorphosis results in the final sex-specific phenotypes (Epper, 1983b; Taylor, 1989a). The male primordium is located in the anterior to lateral region of the disc, in males this region differentiates into the set of internal and external structures of the male reproductive system (Epper and Nöthiger, 1982; Taylor, 1989a). In females the cells of this region divide very slowly during the larval stage and eventually die during metamorphosis (Epper and Nöthiger, 1982; Epper, 1983b; Taylor, 1989a). The female primordium occupies the ventral region of the disc, in females it develops into the external and internal genitalia; in males this region degenerates (Epper and Nöthiger, 1982; Epper, 1983b; Taylor, 1989a). The single anal primordium is located in the posterior-most region of the disc. In females this region gives rise to a dorsal and a ventral anal plate. In males this same region, or a subset of this region, gives rise to a pair of lateral anal plates (Epper and Bryant, 1982; Taylor, 1989a).

The precursors for the genital muscles could either be associated with the genital disc or invade from the terminal abdominal segment, or both, as these sources of myoblasts may not be mutually exclusive. In this chapter I will describe two sets of *twist*-expressing cells in the discs of third instar larvae: one set associated with the male primordia and a second set associated with the female primordia. I propose that these sets are likely candidates for the precursor cells of at least some of the genital muscles. Interestingly, I find no *twist*-expressing cells associated with the anal

primordia. In the last chapter the development of the genital muscles was shown to be under the control of the *doublesex* gene. To further investigate the action of *dsx* in the development of these muscles I have examined the *twist*-expressing cells in third instar discs from flies expressing *dsx*-dominant mutations.

Materials and Methods:

Drosophila stocks

Genital discs were examined from larvae from fly lines used in the previous study of adult genital muscle expression. The P-element line, *P[79B actin-lacZ, ry$^+$]* was used as the wildtype (line 72-3, kindly supplied by Dr. S. Tobin). No difference was found in the pattern of *twist*-expressing cells between discs removed from flies from line 72-3 and the few discs examined from Canton-S flies.

I used dominant mutant alleles of the *doublesex* gene to examine *twist*-expressing cells in the genital discs of larvae with intersexual development. Larvae that were mutant for a *dsx*-dominant allele were generated by crosses between either y^+ *B^{s}Y;dsx^D Sb e^s/TM6B, Tb Hu e^s ca* (*dsx^D/TM6B*) or *B^{s}Y;dsx^M/TM6B, Tb Hu e^s ca* (*dsx^M/TM6B*) males (provided by Drs. B.S.Baker and R.Nagoshi) and females from the 72-3 line. XX; *dsx^D* and XX; *dsx^M* heterozygotes were *Tb$^+$* and *B^{s+}*. Descriptions of the *dsx* and other visible marker alleles used appears in Lindsley and Zimm (1992).

Visualization of *twist*-expressing cells in genital discs

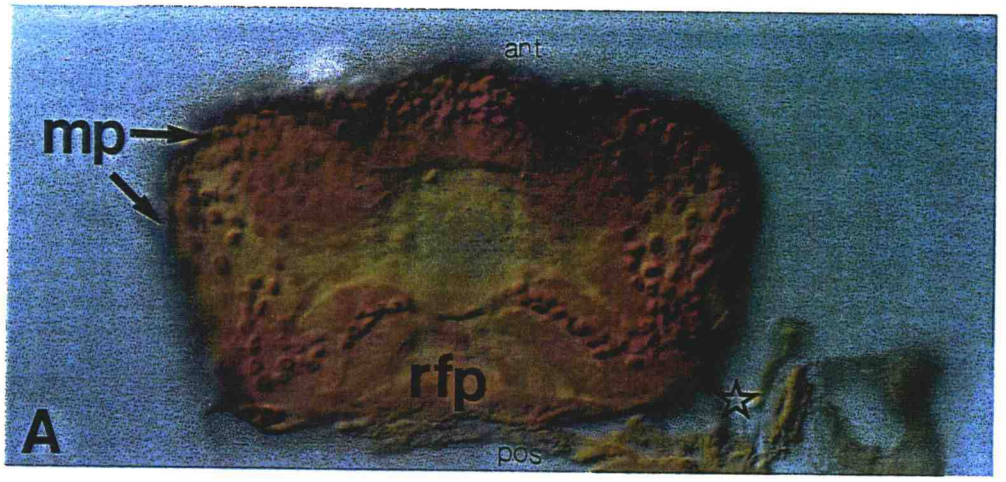
Cells expressing the *twist* protein were visualized using a anti-*twist* antibody (Dr. B. Paterson). Discs from staged animals were dissected away from the rest of the abdomen and fixed in 4% paraformaldehyde (4% PFD) in PBS for 1 hour and then rinsed in PBS. Discs were blocked in 10% heat inactivated normal-goat-serum (NGS) in PBS for 1 hour and incubated for 24 hours in anti-*twist* antisera at a dilution of 1:5,000 in 0.1M PBS containing 0.1% Triton-X and 2% heat-inactivated normal goat serum (PBS-TX-NGS). Following the primary antibody incubation, the abdomens were incubated in a biotinilated secondary antibody (Vectakit, Vector Laboratory) at a dilution of 1:200 in PBS-TX-NGS. Finally, abdomens were incubated in ABC (Vector Laboratory). Diaminobenzidine (DAB; Sigma), in the presence of ß-D-glucose and glucose oxidase in 0.1 M Tris buffer (pH 8.2), was used as the chromogen (Metcalf, 1985). The discs were mounted in Permount resin between two cover slips. In early experiments the discs were left attached to the larval carcass, but removal from the carcass was found to improve staining and resolution of the *twist*-positive myoblasts, especially those associated with the female primordia.

Figure 3.1 Photomicrograph of genital discs from third instar larvae. Presumptive myoblasts were stained using an *α-twist* antibody (see Materials and Methods).

3.1A XY; dsx^+ third instar genital disc. *twist*-expressing cells are found associated with the male genital primordia (MP), the repressed female genital primordia (RFP) and the connection of the genital disc to the CNS (marked with an open star). Note, especially, the *twist*-expressing cells associated with the region of the male primordia that will develop into the genital arch (GA) and lateral plate (LP).

3.1B XX; dsx^+ third instar genital disc. *twist*-expressing cells are found associated with the female genital primordia (FP), the repressed male genital primordia (RMP) and the connection of the genital disc to the CNS (marked with an open star). Note the lighter staining for the *α-twist* antibody in the cells associated with the female primordia.

3.1C XX; *dsxDom*/dsx^+ third instar genital disc. *twist*-expressing cells are found associated with the female genital primordia (FP), the male genital primordia (MP) and the connection of the genital disc to the CNS (marked with an open star).



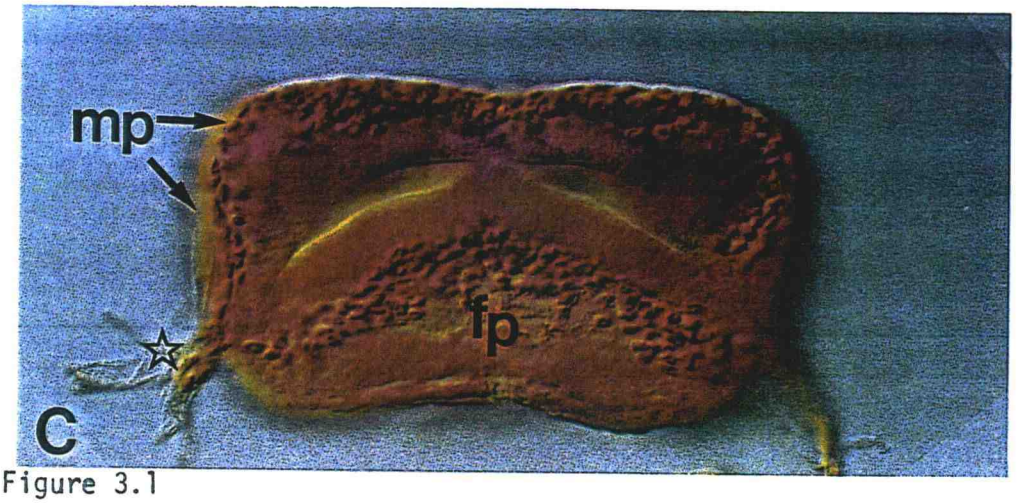
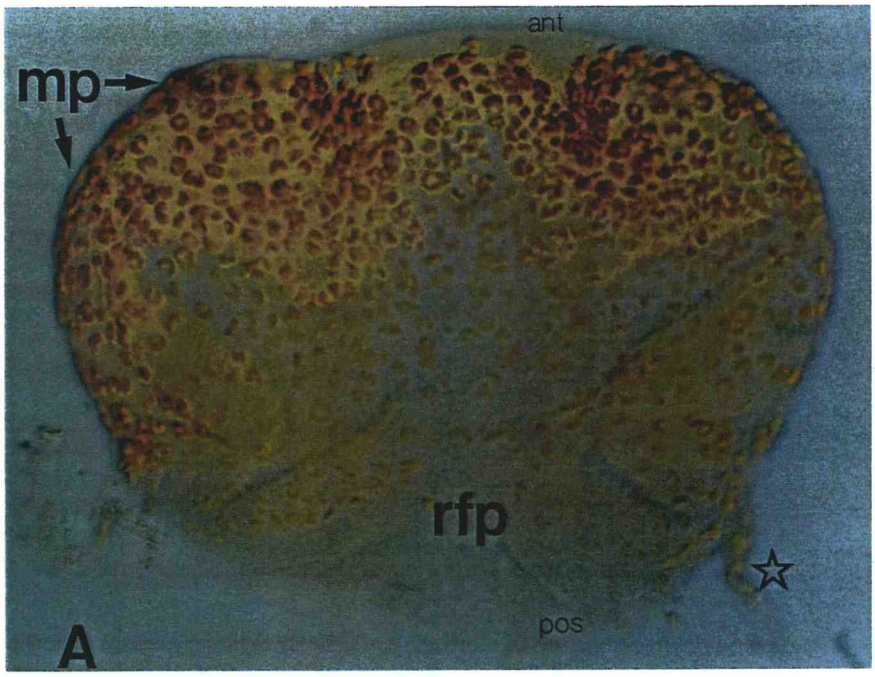


Figure 3.1

Figure 3.2 Photomicrograph of genital discs from white pre-pupae. As in Figure 3.1, presumptive myoblasts were stained using an *α-twist* antibody (see Materials and Methods).

3.2A XY; *dsx*$^+$white pre-pupae genital disc. The segregation of *twist*-expressing cells into two separate groups is less apparent than in the younger discs (Figure 3.1). Cells expressing the *twist* protein are still associated with the male and repressed female, genital primordia, and connection of the disc to the CNS (marked with star), but have begun to spread out across the disc epithelium.

3.2B XX; *dsx*$^+$ white pre-pupae genital disc. As in the male disc, the *twist*-expressing cells have begun to spread across the disc epithelium and blur the edges of the two groups of cells seen in the third instar discs. The repressed male primordium has shrunken to a thin band across the upper (in this view) edge of the disc, note that *twist*-expressing cells still fill this area.



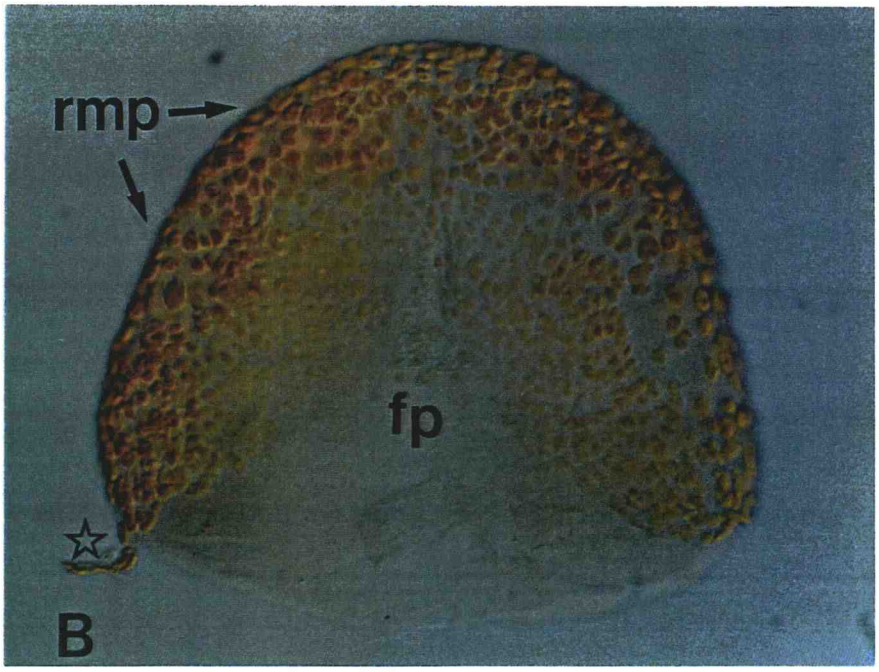


Figure 3.2

Results

twist-expressing cells are found associated with the genital disc of wildtype larvae

I examined discs from both male and female wildtype wandering third instar larvae. The wandering third instar stage is the period during the last larval stage when animals have been committed to enter metamorphosis. Given the association of the *twist*-expressing muscle precursor cells and imaginal discs in the thorax, I had expected to find a similar association of *twist*-expressing cells and the genital imaginal disc. The genital disc, however, is unique in its organization from other imaginal discs, in containing both active and repressed primordia. Since in each genital disc there is a repressed genital primordium, epithelial cells which divide more slowly and an actively dividing, unrepressed primordia, it was possible that the differences in the myoblast population would reflect this differential potential within a given genital disc. In addition, each genital disc has an active anal primordium that might have associated myoblasts.

I found *twist*-expressing cells to be associated with both genital primordia in discs of either sex, but never with the anal primordia (Figure 3.1A). In the genital discs from XY larvae, a large number of *twist*-expressing cells were found along the anterior and lateral regions internal to the epithelium of the male primordium. Additionally, another large and distinct group of *twist*-expressing cells were also present around the ventral region of the repressed female primordium of the disc. *twist*-expressing cells were also present along the lateral projections of the disc that

attach it to the CNS, it was not possible to tell if these groups of cells were continuous with the male of the female groups, or both, or neither. One feature of the antibody labeling was that myoblasts associated with the female primordium were more lightly labeled with the α *twist* antibody than were the myoblasts in the male primordium. No *twist*-expressing cells were found in the region of the anal primordia.

In genital discs from XX larvae, two populations of *twist*-expressing cells were also present (Figure 3.1B). As in XY genital discs, one group of *twist*-expressing cells was located in the anterior and lateral regions of the male primordia and the other group in the ventral part of the female primordia. *twist*-expressing cells were also found along the connection of the XX genital disc to the CNS. As was found for the XY discs, myoblasts associated with the female primordium labeled more faintly than those associated with the male primordia. No *twist*-expressing cells were found associated with the anal primordia.

In addition to discs from wandering third instar larvae, I also examined a limited number of discs from white pre-pupal males and females (Figure 3.2). At this stage of development, larvae have just begun transformation into pupae. In both male and female genital discs of this stage, the separation of the male and female groups of *twist*-expressing cells seen in the wandering third instar discs, was no longer present as the myoblasts began migrating to form a near continuous sheet across the epithelium of the disc.

twist-expressing cells are found associated with the genital disc of larvae expressing *dsx*-dominant mutations

Females heterozygous for a *dsx*-dominant mutation and a wildtype allele ($dsx^{DOM}/+$) develop as intersexes expressing both male and female genitalia, but only one half of the genital muscles seen in wildtype flies (see Chapter 2). Examination of the genital discs from these mutant larvae revealed that expression of both male and female genitalia resulted from a failure to repress the development of the male genital primordia; the discs from third instar XX;$dsx^{DOM}/+$ larvae show a well developed male, as well as, female primordia (Epper, 1981). The reason for the reduction in the number of muscles is unknown, but could be either a result of distortion of cuticular muscles insertion points or a limited number of myoblasts available for formation of the genital muscles (see Chapter 2).

My first step in investigating the effect of *dsx* on the myoblasts that will form the genital muscles was to characterize the *twist*-expressing cells in the genital discs of XX;$dsx^{DOM}/+$ heterozygotes (Figure 3.1C). *twist*-expressing myoblasts, were associated with both the male and female primordia of these discs, in the same region as in the wildtype discs, but not with the anal primordia. From a qualitative analysis it does not appear that the numbers of myoblasts were grossly different between the *dsx*-dominant genital discs and wildtype XY and XX discs. Staining of the myoblasts associated with the female primordium was lighter than those associated with the male primordium, as found in the XX and XY wildtype discs.

Discussion

Pattern of *twist*-expressing cells in genital discs from wildtype male and female discs

In discs from XY and XX third instar larvae, labeling for the *twist* protein reveals a group of cells associated with the male genital primordium and a second, more faintly stained group associated with the female primordium (Figures 3.1A, 3.1B). Not all of these cells are likely to be the precursor myoblasts for the male and female genital muscles, the mgms and fgms, characterized in the last chapter. The male and female primordia develop into the internal genitalia as well as the external genitalia. Many of the *twist*-expressing cells found in these discs probably contribute to the smaller, visceral, muscles that surround and attach exclusively to the internal genitalia. However, some of the *twist*-expressing cells are located over areas of the disc epithelium known to develop into the external genitalia (see Taylor, 1989a) and are therefore likely candidates for the precursors of the mgms and fgms.

The presence of two discrete groups of myoblasts in the larval genital disc at first seems to argue against the proposal that the genital muscles develop from a single group of cells. If the *twist*-expressing cells found in the genital disc are the precursor myoblasts for the genital muscles and act, effectively, as a single primordium, myoblasts from both groups would have to persist and act as a unit to form the mgms from a male genital disc and the fgms from a female genital disc. In fact, by the white pre-pupal stage (the start of puparation) the two groups have merged, forming one continuous group of myoblasts across the disc epithelium (Figure 3.2). This does

not prove that the myoblast do actually all persist, it is still possible that the myoblasts associated with the repressed primordium will degenerate at a later time, but, qualitatively, this is consistent with the myoblasts in the genital disc acting as a single unit.

A quantitative argument for or against a single primordium could be made by comparing the number of myoblasts within a primordium in male and female genital discs. If muscles develop from a single primordium, genital discs from either sex would be expected to contain a similar number of precursor myoblasts. Further, a similar number of *twist*-expressing cells should be found in either primordia, irrespective of the sex of the larvae. That is, the male primordium should have a similar number of *twist*-expressing cells associated with it whether the disc was from an XX or XY larvae; a similar case can be made for the myoblasts associated with the female primordium.

One other important source of myoblasts needs to be considered as it is possible that only a fraction of the mgms and fgms derive from myoblasts associated with the genital disc: the pools of myoblasts found in every abdominal segment (Bate et al., 1991). The lateral muscle in males, mgm10, does not insert on the genitalia, but lies completely within the sixth abdominal segment. If mgm10 does develop from genital disc myoblasts, this represents a migration by these myoblasts over a considerable distance. This muscle could develop from the presumably spatially more immediate myoblasts within the A6 segment, perhaps the dorsal and/or ventral segmental myoblasts. Other muscles, like mgm6, mgm8, fgm5 and fgm7, though attaching to

the external genitalia, resemble slightly modified versions of segmental muscles and also may derive from the segmental pools of myoblasts known to give rise to other segmental abdominal muscles (Bate et al., 1991). *twist* expression is known to overlap expression of muscle-specific proteins (Currie and Bate, 1991). It should be possible, therefore, to follow the development of individual muscles from *twist*-expressing cells in the disc through to adult muscles. Such a developmental time-course would address both the issue of a single or dual primordia and the source of individual muscles.

It is worth noting that no *twist*-expressing cells were found associated with the anal primordium. Muscles associated with the anal plates must be either derived from myoblasts in the populations of myoblasts associated with the male of female primordia or from myoblasts invading from outside the disc.

pattern of *twist*-expressing cells in *dsx*-dominant mutants

Dominant mutations of the *dsx* gene result in expression of substantial elements of both male and female genitalia. In the last chapter we showed that only a subset of the possible male and female genital muscles developed in association with the male and female genitalia of dsx^{Dom} intersexes, a subset containing a total number of around ten muscles, similar to the number found in each sex. I show here that in dsx^{Dom} mutants *twist*-expressing cells are associated with both the male and female genital primordia, as in wildtype genital discs (Figures 3.1A and 3.1B and 3.1C).

In the last chapter, I proposed that the number of the genital muscles expressed in *dsx* mutant intersexes was limited, not by a lack of appropriate insertion sites, but by a finite number of available myoblasts. In this argument, all available myoblasts are required for the development of one complete set of genital muscles. In these intersexual flies, which possess both male and female genitalia, the single pool of myoblasts would be divided amongst the possible male and female muscles by some unknown mechanism. If our proposal is correct then XX; dsx^{Dom}/+ larvae would be expected to a have a similar number of sex-specific myoblasts as wildtype larvae. More specifically, the number and distribution of *twist*-expressing cells that give rise to the mgms and fgms would be expected to be similar between *dsx* mutant and wildtype discs. Indeed, the distribution and relative *twist*-staining properties of myoblasts aligned with the male and female primordia in the XX; dsx^{Dom}/+ genital discs were similar to that in wildtype XY and XX genital discs. Without quantitative analysis, it is not possible to determine whether equal numbers of myoblasts are distributed in the two populations in mutant and wildtype genital discs, but casual observation finds obvious differences. However, similarity, or differences of the particular group(s) of myoblasts destined to form the external genital muscles might easily go unnoticed against a background of myoblasts destined for the internal genital, these muscles do not appear to be drastically reduced in the *dsx* intersexes. Irrespective of the complications in separating fgm and mgm precursor myoblasts from the other *twist*-expressing myoblasts, no counts of *twist*-expressing cells have been done.

Future experiments which would address these quantitative questions and follow the development of the genital muscles, from *twist*-expressing myoblasts, will clarify these points.

Chapter 4.

A Summary of Results and Conclusions

Thomas J. S. Merritt

This project had two main goals: one, to investigate the role, if any, of the *dsx* gene in the development of the genital musculature of *Drosophila melanogaster*, and two, to identify possible developmental precursors of these genital muscles.

In *Drosophila*, development of individual sex-specific characteristics is under the regulation of either of two genetic pathways (for recent reviews see Baker and Ridge, 1980; Baker and Belote, 1983; Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990; Belote, 1992; Burtis and Wolfner, 1992; Cline, 1993). One of these pathways is *dsx*-dependent; the *dsx* gene functions as the output gene of the pathway. The other pathway is independent of the *dsx* gene; its output gene is unknown. The development of the cuticular structures of the genitalia is under the control of the first pathway (Baker and Ridge, 1980). The development of the only previously studied sex-specific muscle, the male-specific MOL, is under control of the second pathway (Taylor, 1992). I found that the development of the sex-specific muscles associated with the genitalia and terminal segments is, similar to the cuticular structures, dependent on *dsx* activity.

In *Drosophila*, adult muscles develop *de novo* from precursors set aside in the embryo; these precursor cells can be identified by their expression of the *twist* protein throughout the larval stage (Bate et al., 1991; Currie and Bate, 1991). I have identified *twist*-expressing cells associated with the male and female primordia of the genital discs of third instar larvae, the imaginal tissues that give rise to the adult male

and female genitalia, respectively. These two groups of *twist*-expressing cells are qualitatively similar in male, female and intersexual dsx^{Dom} discs.

Based on these two main findings, and data generated in forming them, I have drawn several other conclusions, observations and speculations. Here, briefly, is a summary of the main results and conclusions of the preceding chapters.

1) Adult male and female *Drosophila melanogaster* each possess a set of 10 muscles attached to the terminal segments and external and internal genitalia. The set of muscles found in males was morphologically distinct from the set in females, though at least some of the muscles appear to be homologous.

2) In flies possessing a reporter gene construct in which the *lacZ* gene is driven by the *Drosophila 79B actin* gene promotor (*P[79B-lacZ]*), a subset of those muscles express ß-galactosidase by midway through adult development. In males, six of the genital muscles stained for ß-galactosidase. In females, seven of the genital muscles stained for ß-galactosidase. The only other abdominal muscle showing this early pattern of reporter expression was the Muscle of Lawrence (MOL), the other known sex-specific muscle.

3) Proper development of the male and female genital muscles is dependent upon normal *dsx* activity. Both XX; dsx^- and XY; dsx^- intersexes developed the same set of genital muscles. In contrast, MOL development is *dsx*-independent; XY; dsx^-, but not

XX; *dsx*$^{-}$, intersexes develop a MOL.

4) *dsx*$^{-}$ intersexes develop a subset of the genital muscles seen in wildtype males and females. This subset contained both distinctly male and distinctly female muscles, but no intersexual muscles. In total, about one half (between seven and thirteen) of the total possible male and female genital muscles (twenty) develop in the intersexes. This limited number of expressed genital muscles may result from development from a limiting pool of muscle precursor cells.

5) Expression of the *P[79B-lacZ]* reporter gene is a separate sex-specific phenotype from muscle expression. Genital muscles that stained for ß-galactosidase in wildtype flies did not always stain in either *dsx*$^{-}$ or *dsx*^{Dom}/+ intersexes. Staining of most muscles ranged between less than 50 and 100%; one muscle, fgm10, never stained. This general reduction in staining frequency may reflect a failure of these muscles to fully differentiate into their sex-specific phenotype.

6) Muscle fibers spanning between the A7 tergite and sternite sometimes stained for activity of the *P[79B-lacZ]* reporter gene construct. The staining may be a product of transformation of the muscle fibers into a male-like (mgm10) phenotype. This ectopic muscle staining is much more frequent in *dsx*^{Dom}/+ intersexes than in *dsx*$^{-}$ intersexes.

The higher frequency of transformation (staining) may represent the activation of a male phenotype by the ectopic presence of the DSX^{M} protein.

7) The product of the *dsx* gene acts as a negative regulator in the development of the genital muscles, more similar to its role in the cuticle (Baker and Ridge, 1980) than its role in the MOL (Taylor, 1992). The same set of genital muscles found in the , loss-of-function mutants, the dsx^{-} intersexes, is found in gain-of-function *dsx* mutants, the $dsx^{Dom}/+$ intersexes.

8) Two groups of putative myoblasts, identified by their expression of the *twist* protein, are present in the genital discs of male and female wandering third instar larvae. One group is located in the male primordia and the other in the female primordia. These are likely candidates for the developmental precursors of the genital muscles. No *twist* expressing cells are located in the region of the anal primordia. These two groups of cells are qualitatively similar in discs from XX, XY and XX; $dsx^{Dom}/+$ animals.

9) The physical division of the two groups of putative myoblasts is no longer apparent by the white-prepupal developmental stage. This, and the similarity of the groups across genotypes suggests that the myoblasts act as a single primordia for both the male and female muscles.

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Appendix

Appendix.
Creating fly lines of interest

Construction of a P[79B-lacZ] *dsx*^{1} recombinant chromosome

Genital muscles of control flies were mapped and identified using differential interference contrast optics and staining for ß-galactosidase in the P[79B actin-lacZ, *ry*$^{+}$] (*P[79B-lacZ]*) line. To facilitate identification of similar muscles in *dsx* intersexes, I needed to construct *dsx*$^{-}$ mutants that also had the *P[79B-lacZ]* insert. I approached this problem in two parts; location of the insert and construction of a *P[79B-lacZ] dsx*$^{-}$ chromosome.

Because *dsx*$^{-}$ alleles are recessive mutations both chromosomes need to have mutant alleles for a mutant phenotype to be expressed. If the insert was on the second chromosome then it would be possible to generate *P[79B-lacZ];dsx*^{1}/*dsx*^{1} or *P[79B-lacZ];dsx*^{1}/*Df(3R)dsx15* flies through simple crosses. If, however, the insert was on the third chromosome we would have to generate a *P[79B-lacZ],dsx*^{1} chromosome through meiotic recombination. The initial published account (Courchesne-Smith and Tobin, 1989) placed the *P[79B-lacZ]* insert in the 72-3 line at 70C, on the left arm of the third chromosome, which on the meiotic maps is at 3-41.

I used simple crosses to definitively locate the insert in the line that I used for the muscle investigations. I crossed *P[79B-lacZ]* homozygotes to a double balancer line, and crossed the F1 heterozygotes to a test line, scoring the F2 generation for the *P[79B-lacZ]* insert and balancer chromosomes. Balancers, chromosomes with

multiple inversions, prevent chromosomes in which recombination has occurred from being present in oocytes, and were used to prevent recombination between chromosomes carrying different markers. Flies heterozygous for balancer chromosomes can be identified by the presence of dominant, visible markers. In this genetic test, consistent segregation of the visible marker within the p-element construct from a test balancer chromosome would indicate that the P element was on the chromosomal homologue of the test balancer; lack of consistent segregation would indicate that the insert and marker were not on the same chromosome.

A test for the presence of the P-element construct on the second and/or third chromosome

In the parental cross (figure A.1), *P[79B-lacZ]/P[79B-lacZ]* flies were crossed to *TM2,ry*/MKRS flies. *TM2* and *MKRS* are both third chromosome balancers. *TM2* carries a dominant *Ultrabithorax* allele (Ubx^{130}); a haltere marker. *MKRS* carries Stubble (*Sb*); a dominant bristle marker. Additionally, both of these balancers were mutant for the *rosy* (*ry*), which effects eye color; the P-element carries a ry^+ gene as a visible marker.

If the insert was on the third chromosome then an F2 fly would never contain both *P[79B-lacZ]* and the balancer. If the insert was on the second chromosome then both the balancer and the insert would be present in approximately 25% of the flies. The insert was followed in two ways. Only flies carrying the insert would have muscles expressing ß-galactosidase, which can be detected by incubation in the X-gal reagent. Additionally, a more immediate, less invasive, method was possible; the *P[79B-lacZ]*

insert carries a ry$^+$ allele, so that only flies with the *P[79B-lacZ]* insert *ry*$^+$. As well as being faster than scoring by staining for ß-galactosidase, scoring for the insert by the *ry*$^+$ phenotype does not require killing the flies, while not a concern in these crosses, but in later crosses becomes important.

Parental cross: *P[79B-lacZ]/P[79B-lacZ]* (x) *TM2, Ubx*^{130} *ry/MKRS Sb ry*

[select *P[79B-lacZ]/MKRS*]

F1 cross: *P[79B-lacZ]/MKRS* (x) *ry*^{506}/*ry*^{506}

[score F2 flies for *P[79B-lacZ]* and *Sb*]

Figure A.1

Of 39 F2 flies from the F1 cross, 20 were *P[79B-lacZ]*, *ry*$^+$ and 19 were *Sb*. No flies were found that were both *P[79B-lacZ](ry*$^+$*)* and *Sb*. The insert segregated from the balancer; it is on the third chromosome.

To double-check this result I repeated the experiment, crossing *P[79B-lacZ]/P[79B-lacZ]* flies to *In(2LR)Gla/CyO* flies (Figure A.2). *In(2LR)Gla* is an inversion, marked with the *Glazed* (*Gla*) gene, an eye morphology marker, that suppresses crossing over on the second chromosome. *CyO* is a second chromosome balancer marked with the Curly (*CY*) gene, a wing morphology marker. Because all

third chromosomes in these flies were ry^+, presence of the *P[79B-lacZ]* insert could only be detected by staining for ß-galactosidase.

Parental cross: *P[79B-lacZ]/P[79B-lacZ]* (x) *In(2LR)Gla/CyO*

[select *P[79B-lacZ]/In(2LR)Gla*]

F1 cross: *P[79B-lacZ]/In(2LR)Gla* (x) Canton-S

[score F2 flies for *P[79B-lacZ]* and *Gla*]

Figure A.2

Of the 41 flies scored for *P[79B-lacZ]* and *Gla* , 9 were *Gla* alone, 7 were *P[79B-lacZ]* alone, 13 were neither Gla nor *P[79B-lacZ]* and 12 were both *Gla* and *P[79B-lacZ]*. The insert did not segregate consistently from the second chromosome markers. The insert is not on the second chromosome, thus I had to generate a recombinant *P[79B-lacZ]*, dsx^1 chromosome.

Recombinant crosses to construct the P[79B-lacZ], dsx^1 chromosome

To do this I needed to be able to score for the presence of the *P[79B-lacZ]* insert without having to kill and stain the fly. The one way to do this is to follow the insert using the ry^+ marker gene, as I did in the crosses to *TM2/MKRS*, which necessitates a

ry⁻ background. No *dsx*⁻, *ry*⁻ chromosomes were available from the stock centers. I began by creating a *ry*⁻ third chromosome, with markers to allow us to follow recombinations in the region of the *dsx* gene, through meiotic recombination (Figure A.3).

Parental cross: ry^{506}/ry^{506} (x) *ru h st* p^p *cu sr* e^s/*ru h st* p^p *cu sr* e^s

[select virgin females]

F1 cross: XX:; *ru h st* p^p *cu sr* e^s/ry^{506} (x) *ru h st* p^p *cu sr* e^s/*ru h st* p^p *cu sr* e^s

[select for the recombinant *sr*,e^s]

F2 cross: *sr* e^s/*sr* e^s (x) *TM2/MKRS*

[select *ry*⁻ line and maintain; these flies are ry^{506} *sr* e^s/*TM2*]

Figure A.3

ry^{506} homozygotes were crossed to a line containing multiple recessive markers to create heterozygotes (Figure A.3). The females are the flies that were used to produce the recombinants; that is, gametes in which the ry^{506} and *ru h st* p^p *cu sr* e^s chromosomes that had paired, crossed over and recombined to create a ry^{506},sr,e^s chromosome. Females were selected because recombination does not occur in male

Drosophila. The female heterozygotes were back crossed to *ru h st p^p cu sr e^s/ru h st p^p cu sr e^s* males to score for the loss of the markers proximal to the *ry* gene, indicating recombination (Figure A.3). Not all of the recombinants were ry^{506}, since *sr* and *ry* are 10 map units apart. To find the ry^{506} recombinants all male *sr e^s* flies were crossed to *TM2/MKRS* virgin females (Figure A.3). *sr e^s* males were selected since they do not have meiotic recombination; preventing recombination of the ry^{506} *sr e^s* and *ru h st p^p cu sr e^s* chromosomes.

My next step was to recombine dsx^1 onto the ry^{506} *sr e^s* chromosome (Figure A.4). Both XX and XY dsx^1 homozygotes are infertile so I used the closely linked gene p^p to follow dsx^1 (dsx^1 and p^p are separated by only .1 map units), while maintaining a fertile line.

ry^{506},*sr*,e^s/*TM2* males were crossed to p^p dsx^1 females to generate heterozygotes, p^p dsx^1/ry^{506} *sr e^s*, that were to form the recombinants (Figure A.4). Recombinants were recovered as p^p *sr e^s* flies, effectively all of which were p^p dsx^1 *sr e^s*. I used the *ru h st p^p cu sr e^s* chromosome, which has p^p, to follow the dsx^1 gene. This does not, however, allow me to score for ry^-. Individual recombination events could have been either proximal or distal to the *ry* locus, only proximal recombinations would give me a p^p dsx^1 ry^{506} *sr e^s* chromosome. To test for the presence of ry^{506}, all p^p *sr e^s/ru h st p^p cu sr e^s* males were crossed to the *TM2/MKRS* line (Figure A.4). Again, males were used to prevent further recombination of the p^p dsx^1 *sr e^s* chromosome.

Recombination of the *P[79B-lacZ]* insert onto this dsx^1 chromosome could be followed using the ry^+ phenotype. Finally, I crossed dsx^1 ry^{506} flies to *P[79B-lacZ]*

homozygotes and then crossed the resulting female heterozygotes, XX;p^p dsx^1 ry^{506} s^r e^s/*P[79B-lacZ]*, to the ry^{506} *sr* e^s line to score for ry^+ recombinants (Figure A.5).

The ry^{506} *sr* e^s chromosome allowed me to score for recombinants that had picked up the insert; only these flies were ry^+ *sr* e^s. Using this chromosome I was not able to score for p^p dsx^1, but only recombinants proximal to p^p would give me the *P[79B-lacZ]*, dsx^1 chromosome. To score for p^p, and thereby dsx^1, all male ry^+ *sr* e^s flies were individually crossed to virgin female p^p dsx^1 ry^{506} *sr* e^s/*TM2*. All lines that produced dsx^- intersexes had both the *P[79B-lacZ]* insert and the dsx^1 gene.

Parental cross: ry^{506} *sr* e^s/*TM2* (x) p^p dsx^1/*TM6b*

[select Ubx^{130+}, Tb^+ virgin females]

F1 cross: XX;p^p dsx^1/ry^{506} *sr* e^s (x) *ru h st* p^p *cu sr* e^s/*ru h st* p^p *cu sr* e^s [select p^p,*sr*,e^s]

F2 cross: XY;p^p dsx^1 *sr* e^s/*ru h st* p^p *cu sr* e^s (x) *TM2/MKRS*

[select ry^-: p^p dsx^1 ry^{506} *sr* e^s/*TM2*]

Figure A.4

Two lines were found to have the *P[79B-lacZ] p^p dsx^1 ry^{506} sr e^s* chromosome. These were maintained over the balancer *TM2*.

Parental cross: *p^p dsx^1 ry^{506} sr e^s/TM2* (x) *P[79B-lacZ]/P[79B-lacZ]*

[select virgin female *Ubx^{130+}*]

p^p dsx^1 ry^{506} sr e^s/P[79B-lacZ] (x) *ry^{506} sr e^s/TM2*

[select *ry^+ sr e^s*]

XY;*P[79B-lacZ] ry^{506} sr e^s/ry^{506} sr e^s* (x) *p^p dsx^1 ry^{506} sr e^s/TM2*

[select *dsx^1 ry^+*]

Figure A.5

Creating a y/y$^+$Y;P[79B-lacZ] *dsx*^{1} line:

I now had the *P[79B-lacZ] dsx^1* line I set out to create. Homozygotes were intersexual with staining genital muscles. In addition to comparing muscles between controls, dominant *dsx^1* mutants and recessive *dsx* mutants I was also interested in comparing XX and XY recessive *dsx* mutants. I could distinguish XX and XY *dsx* intersexes in three ways. These intersexes are phenotypically very similar, but can be distinguished by expression of a fifth segment male-specific muscle, the Muscle of

Lawrence (MOL). MOL expression is independent of *dsx* control;XY, but not XX, *dsx* mutants express a MOL. In addition I used a chromosomal indicator of sex.

Parental cross: *y*/y$^+$Y;*p^p dsx^1*/TM6 (x) X/Y;*TM2/MKRS*

[select *y*/X;*p^p dsx^1/TM2* virgins and X/*y*$^+$Y;*MKRS/p^p dsx^1*]

F1 cross: *y*/X;*p^p dsx^1/TM2* (x) *y*/*y*$^+$Y;*p^p dsx^1/TM6B*

[select *y*/*y*;*p^p dsx^1/TM2* virgin females]

F2 cross: *y*/*y*;*p^p dsx^1/TM2* (x) X/*y*$^+$Y;*MKRS/p^p dsx^1* (from the first cross)

[select *y*/*y*$^+$Y;*TM2/MKRS*]

F3 cross: *y*/*y*$^+$Y;*TM2/MKRS* (x) X/*y*;*TM2/MKRS*

[select *y*/*y*;*TM2/MKRS*]

F4 cross: *y*/*y*;*TM2/MKRS* (x) *y*/*y*$^+$Y;*TM2/MKRS* (from the third cross)

[maintain as a line]

Figure A.6

Two different tactics were used. In creating the *dsx^1*1/*Df(3R)dsx15* transheterozygote I crossed XY;*P[79B-lacZ] dsx^1/TM2* flies to *y*/*y*;*Df(3R)dsx15/TM6B*

flies. *y*,yellow, is a cuticle pigment marker. All F1 XY flies were *y*/Y and therefore yellow; XX flies were *y*/X and had wild type pigment.

I determined chromosomal sex in *P[79B-lacZ]dsx*^{1}1 homozygotes by creating a *y*/*y*$^{+}$Y line of flies through standard crosses. A *y*$^{+}$Y is carries the wild type yellow gene, normally found on the X chromosome. y/*y*$^{+}$Y males have wild type pigmentation.

I first created a *y*/*y*$^{+}$Y; *TM2*/*MKRS* line from a *y*/*y*$^{+}$Y;*p*^{p} *dsx*^{1}/TM6 line and the *TM2*/*MKRS* line (Figure A.6). In these crosses the *p*^{p} *dsx*^{1}/TM6 chromosomes were not important, this line I used only as a source of the *y* and *y*$^{+}$Y chromosome.

Parental cross: X/X; *P[79B-lacZ] p*^{p} *dsx*^{1} *ry*^{506} *sr e*^{s}/*TM2* (x) *y*/*y*$^{+}$Y;*TM2*/*MKRS* [select virgin female *TM2* flies]

F1 cross: *y*/X;*P[79B-lacZ] p*^{p} *dsx*^{1} *ry*^{506} *sr e*^{s}/*TM2* (x) *y*/*y*$^{+}$Y;*TM2*/*MKRS* [select *y* *TM2* virgin females]

F2 cross: *y*/*y*;*P[79B-lacZ] p*^{p} *dsx*^{1} *ry*^{506} *sr e*^{s}/*TM2* (x) *y*/*y*$^{+}$Y;*TM2*/*MKRS* [select male and female *TM2* and maintain as a line]

Figure A.7

Finally I generated a stable, *y*/y^{+}Y;*P[79B-lacZ] dsx*^{1} line through a series of crosses between the *y*/*y*$^{+}$Y;*TM2*/*MKRS* line and the *P[79B-lacZ] dsx*^{1} lines (Figure

A.7).

This was the last line I generated; a y/y$^+$Y;*P[79B-lacZ] dsx^1* line in which the homozygotes develop as intersexes, distinguishable as XX or XY, with *P[79B-lacZ]* staining. The two *P[79B-lacZ] dsx^1* lines were maintained both as unmarked XY and *y*/y$^+$Y lines.

Crosses involving dominant *dsx* alleles

I also wished to use the *P[79B-lacZ]* insert to aid our identification of genital muscles in flies expressing dominant alleles of *dsx*. Because the dominant *dsx* alleles are expressed in heterozygotes and a single copy of the *P[79B-lacZ]* insert is sufficient for robust staining, a single cross between *dsx^D* males and females homozygous for the *P[79B-lacZ]* insert gave us *dsx* mutants with muscle staining (Figure A.8).

The Y chromosome in these crosses carries the *Bar stone* (*B^s*) allele, an adult eye morphology marker, only XX flies were *B^{s+}*. Additionally, in the line we used *dsx^D* is maintained over *TM6B*, a balancer chromosome with a dominant marker *Tb* (*Tubby*), a body shape marker detectable in all stages of the *Drosophila* life cycle. XX;*P[79B-lacZ] dsx^D* intersexes were *B^{s+} Tb$^+$* and could, therefore, be distinguished from *dsx$^+$* flies as larvae, pupae and adults.

I also looked at the muscles in flies mutant for another dominant *dsx* allele, *dsx^M* mutants, using a similar cross.

Parental cross: B^sY;dsx^D *Sb* e^s/*TM6B,Tb* (x) X/X;*P[79B-lacZ]/P[79B-lacZ]*

[select B^{s+} Tb^+]

Parental cross: B^sY;dsx^M/*TM6B,Tb* (x) X/X;*P[79B-lacZ]/P[79B-lacZ]*

[select B^{s+} Tb^+]

Figure A.8

XX flies heterozygous for a dominant dsx allele and a recessive dsx allele, or a dsx^- deficiency, develop as somatic males. To check that the genital muscles in these pseudomales developed and stained as in wildtype males we crossed dsx^D or dsx^M males to *P[79B-lacZ]* p^p dsx^1 ry^{506} *sr* e^s/*TM2* females (Figure A.9). Pseudomales were identified as Bs^+,Ubx^{130+},Tb^+. Additionally, I checked muscle development, but not staining, in dsx^D/Df(3R)dsx15 pseudomales, generated through a similar cross (Figure A.9)

Parental cross: $B^{s}Y;dsx^{D}$ *Sb* e^{s}/*TM6B,Tb* (x) X/X;*P[79B-lacZ]* p^{p} dsx^{1} ry^{506} *sr* e^{s}/*TM2*

[select *Sb* B^{s+} Ubx^{130+} Tb^{+}]

Parental cross: $B^{sY};dsx^{M}$/*TM6B,Tb* (x) X/X;*P[79B-lacZ]* p^{p} dsx^{1} ry^{506} *sr* e^{s}/*TM2*

[select B^{s+} Ubx^{130+} Tb^{+}]

Parental cross: $B^{s}Y;dsx^{D}$ *Sb* e^{s}/*TM6B,Tb* (x) *y*/*y*;*Df(3R)dsx15/TM6B*

[select *Sb* B^{s+} Tb^{+}]

Figure A.9