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REVIEW

The Developmental Consequences of Alternate Splicing in Sex Determination and Differentiation in *Drosophila*

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INTRODUCTION

Many eukaryotic genes generate alternately spliced transcripts which can produce different proteins or which have altered translational controls. One of the most direct demonstrations that alternately spliced forms of transcripts lead to different developmental consequences lies within the sex determination pathway of *Drosophila*. The *doublesex* (*dsx*) gene at the end of this pathway in somatic cells encodes two differently spliced transcripts, one specific for males and one specific for females (Burtis and Baker, 1989). These encode proteins with a common DNA binding region and a sexually unique carboxy terminus; DSX^M and DSX^F act as transcription factors and have opposing activities. The main developmental consequences are the repression of a set of downstream female-specific differentiation genes by DSX^M in males and the repression of certain male characteristics by DSX^F in females (for reviews see Slee and Bownes, 1990; Steinmann-Zwicky *et al.,* 1990; Ryner and Swain, 1995).

Many of the sexual differences between male and female *Drosophila* are controlled by the two alternate products of the *dsx* gene (Burtis and Baker, 1989). They direct both the determination of sex-specific characteristics in the imaginal cells and the maintenance of determination throughout subsequent cell divisions. This regulation leads to the final differentiation of male or female genitalia, the differences in pigmentation patterns in the abdomen of each sex and in other sex-specific bristle patterns, such as the sex comb on the first leg of the male. Differences between the sexes in the pattern of nerve cell divisions are also directed by the two related DSX proteins (Taylor and Truman, 1992), as is the selection between the male or female development of the gonad into a testis or ovary and its subsequent differentiation (Szabad and Nöthiger, 1992). The *yolk protein* genes which are expressed in the adult female fat body are targets of the DSX protein (Burtis *et al.,* 1991) and their yolk protein products are essential for oocyte development.

However, the use of alternate splicing in sexual development in *Drosophila* is not limited to *dsx.* Alternate splicing of *dsx* transcripts is controlled by the products of the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes (Nagoshi *et al.,* 1988). *tra* RNA is also alternately spliced, in this case with the dramatic consequence that in males no functional protein product is made (Butler *et al.,* 1986; McKeown *et al.,* 1987), whereas in females an RNA binding protein is produced which interacts with the *tra-2*-encoded RNA binding protein (Belote and Baker, 1982), directing the female-specific splicing of *dsx*. It seems likely that *tra* and *tra-2* have other targets in addition to *dsx* that are important for sexual development, since several aspects of sexual dimorphism depend upon the *tra/tra-2* genes but are independent of *dsx* (Taylor *et al.,* 1994). This includes courtship behaviour; the development of abdominal cells, which produce a female pheromone; and the correct innervation of nerves needed for the development of a male-specific muscle (Lawrence and Johnston, 1986; Taylor, 1992).

The female-specific splicing of *tra* RNA is itself directed by an alternately spliced gene product. This is encoded by the *Sex-lethal* (*Sxl*) gene that is at the head of the sex-determination hierarchy (Cline, 1984, 1993). Sxl produces many transcripts, including several specific to female somatic cells, that generate a functional RNA binding protein. The male mRNA from *Sxl* does not encode a functional protein (Bell *et al.,* 1988). The SXL protein is known to direct the female-specific splicing of its own RNAs as well as that of *tra* (Inoue *et al.,* 1990; Bell *et al.,* 1991; Horabin and Schedl, 1993a,b; Samuels *et al.,* 1994).

The female product of *Sxl* also directs suppression of the

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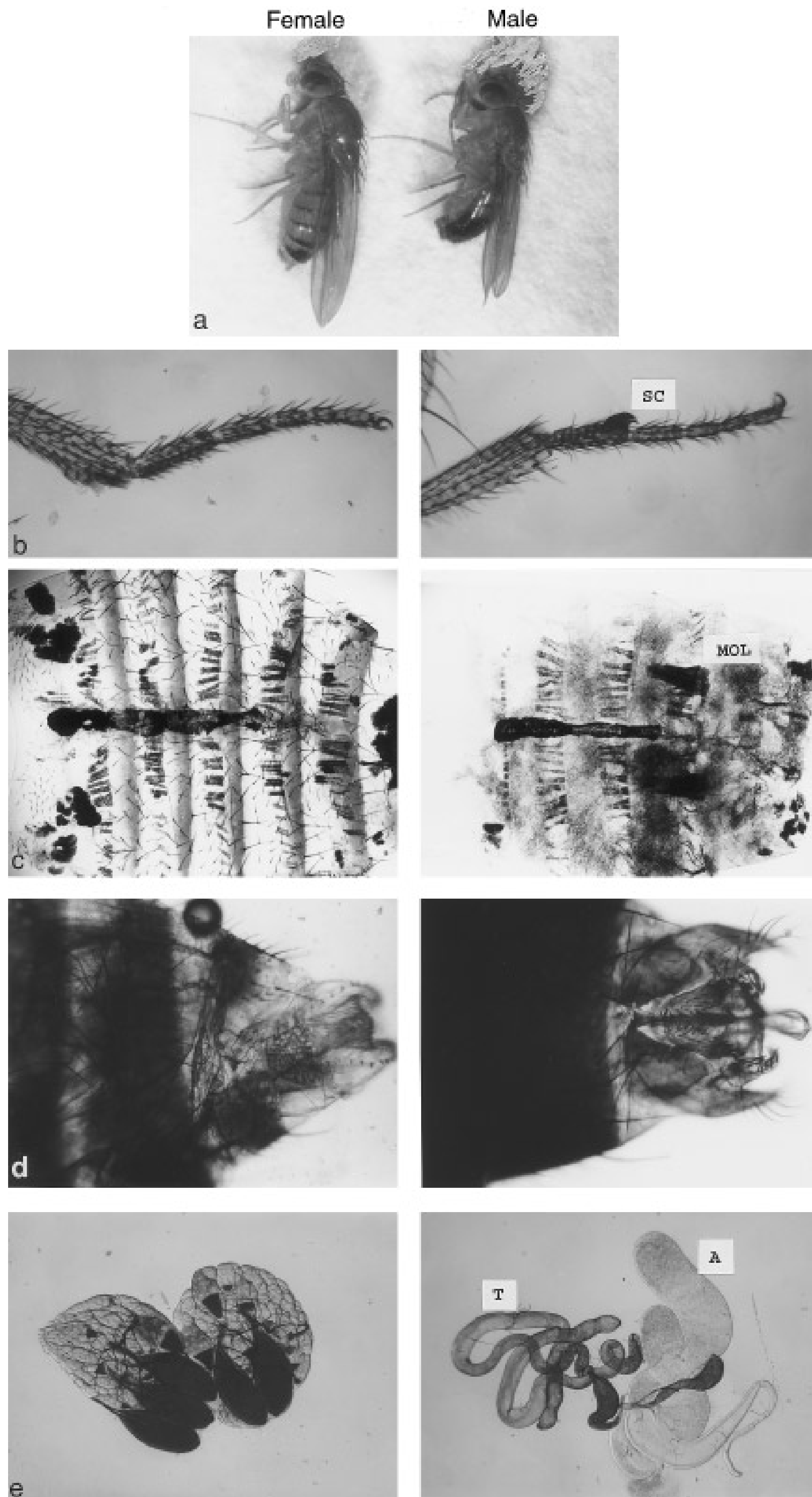
Female
Male
a
b
SC
c
MOL
d
e
T
A

hyperactivation of the X-chromosomes in females (Baker and Belote, 1983; Lucchesi and Manning, 1987; Baker *et al.,* 1994). X-chromosome hyperactivation is a process that is essential for dosage compensation in males, as they carry only one X-chromosome. It is this function of *Sxl* which leads to sex-specific lethality when *Sxl* is inappropriately activated or repressed. Female differentiation of the germline also depends upon *Sxl* (Steinmann-Zwicky, 1992). From its multiple functions, some of which are not mediated through the activities of *tra* and *tra-2,* it seems that *Sxl,* like *tra,* must have more target genes that remain to be discovered, namely those involved in dosage compensation, size (females are larger than males), and germline differentiation. Thus this pathway provides excellent insight into how alternate splicing can be used to ensure that very different developmental decisions are taken during development. Its branched nature, with multiple targets at each point in the hierarchy, shows how a complex network of interrelated processes can be controlled at the level of mRNA processing. Figure 1 shows the morphological sexual differences between adult males and females, and Fig. 2 summarises the sex determination pathway in somatic cells.

SOMATIC SEX DETERMINATION

The primary determinant of sex in flies is the number of X-chromosomes to sets of autosomes, the X:A ratio (see reviews by Slee and Bownes, 1990; Steinmann-Zwicky *et al.,* 1990; Cline, 1993; Ryner and Swain, 1995). Those flies that have one X-chromosome to two sets of autosomes (X:A = 0.5) are male, whilst those that have an X:A ratio of 1 (i.e., two X-chromosomes to two sets of autosomes) are female. The process of somatic sex determination is largely cell autonomous; there appears to be no hormonal component. This can be inferred from studies on gynandromorph flies possessing both XO and XX cells which develop into male and female tissue autonomously. Since the male and female cells are exposed to the same compounds circulating in the haemolymph, a hormonal influence can be excluded. The Y-chromosome does not play a role in sex determination, unlike the process in mammals; rather it carries genes which are required to complete spermiogenesis (Fuller, 1993).

Activation of Sex-lethal

The X:A ratio is assessed by a number of zygotic loci that are located on the X-chromosome. These activate *Sex-lethal* and are known as numerator elements (*sisterless-a* (*sis-a*), *sisterless-b* (*sis-b*), and *runt*). There are also repressor elements (or denominators) on the autosomes (*deadpan* (*dpn*)) (for review see Parkhurst and Meneely, 1994). An additional X-linked locus known as *sisterless-c* (*sis-c*) has also been identified; this appears to act as a numerator element although its effects are weaker than those of either *sis-a* or *sis-b* (Cline, 1993). Numerator elements act as feminising elements, while the known denominator acts as a masculinising element (Cline, 1993). Alterations in the dosage of these elements leads to sex-specific lethality. A reduction in the numerator dose results in female-specific lethality, while an increase is lethal to males (Cline, 1988; Younger-Shepherd *et al.,* 1992). The denominator element exhibits the reciprocal phenotype, an increase in dosage resulting in female lethality. The assessment of the X:A ratio affects the activation of the gene *Sxl*. Cells that have an X:A ratio of 1 (i.e., female) activate *Sxl*, while those that have an X:A ratio of 0.5 (i.e., male) do not activate *Sxl*.

Several of the numerator elements and the denominator element *deadpan* have been cloned and characterised at the molecular level. *sis-a* is a member of a family of transcription factors known as basic leucine zippers (Erickson and Cline, 1993a). *sis-b* (which corresponds to *scute* α/T_4, a member of the *achaete/scute* complex) encodes a basic helix–loop–helix protein (bHLH) (Cline, 1988; Torres and Sánchez, 1989; Erickson and Cline, 1993b). The pair-rule segmentation gene *runt* has also been implicated in sex determination (Duffy and Gergen, 1991). The predicted *runt* protein shows homology with a family of transcriptional regulators, including the polyoma enhancer binding protein. Interestingly, this gene, unlike *sis-a* and *sis-b* which act throughout the embryo, appears to affect sex determination only in the central region of the trunk. To date, only one denominator element has been identified, the proneural gene *dpn,* which encodes a bHLH protein. As expected for a denominator element, alterations in the gene dosage of this locus result in sex-specific lethality (Younger-Shepherd *et al.,* 1992; Cline, 1993).

In common with many other processes in *Drosophila,* sex determination relies upon maternally contributed RNAs laid down in the oocyte during oogenesis. One of these is encoded by the gene *daughterless* (*da*) (Caudy *et al.,* 1988; Cronmiller *et al.,* 1988). As its name suggests, females mutant at one allele of this locus (*da*^{1}) do not produce any female progeny. Again, *da* encodes a protein that contains a bHLH domain. The neural locus *extramacrochaetae* (*emc*) has been predicted to act as a negative regulator of *Sex-lethal.* The protein contains a HLH domain but does not contain the basic residues which are essential for DNA binding (Younger-Shepherd *et al.,* 1992; Bier *et al.,* 1992).

FIG. 1. Morphological differences between males and females. (a) Male and female flies; note the male is smaller than the female. (b) Male and female foreleg; note the sex comb on the male. (c) Male muscle and equivalent segment in female (photographs courtesy of Dr Peter Lawrence, MRC, Cambridge). (d) Male and female genitalia and abdominal pigmentation; note the more extensive pigmentation in the male. (e) Male and female gonad. A, accessory gland; MOL, muscle of Lawrence; SC, sex comb; T, testis.

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In this case, maternally contributed *emc* may act by binding with the other HLH-containing proteins to form nonfunctional heterodimers. Recently, another protein which interacts with hairy-related bHLH proteins has been identified (Paroush *et al.,* 1994). The protein encoded by *groucho,* has been shown to interact with *dpn* and may act as a transcriptional corepressor in conjunction with other bHLH proteins. The *hermaphrodite* (*her*) locus has multiple roles during sex determination. Maternally contributed HER appears to act as a positive regulator of *Sxl* activation and also affects the process of dosage compensation. The zygotic function of *her* is not rescued by the constitutive expression of either *Sxl* or *tra. dsx* splicing is unaffected in intersexual flies resulting from *her* zygotic mutants. This implies that the zygotic function of *her* may be similar to that of *intersex* (*ix*), acting in parallel with or downstream of *doublesex* (Pultz *et al.,* 1994; Pultz and Baker, 1995). *her* has recently been characterised at the molecular level. It encodes a zinc finger protein which may function as a transcription factor (Ryner and Swain, 1995). These proteins activate *Sex-lethal* at the level of transcription (Keyes *et al.,* 1992). The various bHLH proteins are able to interact to produce homo- or heterodimers which then bind to DNA, activating transcription. The ability of these proteins to form heterodimers resulting in *Sxl* activation was demonstrated by the inappropriate expression of another bHLH protein, the pair-rule gene *hairy* (Parkhurst *et al.,* 1990). When *hairy* protein is expressed ectopically under the control of the *hunchback* promoter, it is a female lethal. It was shown that HAIRY interacts with the other HLH proteins to form heterodimers which could not bind to DNA, preventing *Sxl* from being activated in females. The gene *sans fille* (*snf*) (known also as *liz* (Steinmann-Zwicky, 1988) and *fs(1)1621*) represents a maternal effect gene that is required for activation of *Sxl* in both the germline and the soma. *snf* has been characterised at the molecular level and shows significant sequence and functional homology with the U1A snRNP protein (Flickinger and Salz, 1994). The *virilizer* locus has also been implicated in the process of sex determination (Hilfiker and Nöthiger, 1991). However, it is not yet clear where in the hierarchy *vir* acts. It appears to function upstream of *tra* to modulate *Sxl* activity but whether it acts directly on *tra, Sxl,* or both has not been established.

It is interesting to note that several of the genes involved in the assessment of the X:A ratio also have a function later in development, during neurogenesis. The maternal product of *daughterless,* for example, is required during sex determination; the zygotic function is required later for development of the peripheral nervous system (Caudy *et al.,* 1988). *sis-b* (a member of the *achaete/scute* complex), *deadpan* (proneural gene), and *extramacrochaetae* (neural gene) are also involved in both of these processes. These bHLH proteins may interact to constitute a genetic switch, such that the ratio of positive (numerators) to negative (denominators) regulators determines a cell's fate by affecting the transcriptional activity of downstream genes.

Sex-lethal

Sex-lethal plays a pivotal role in the processes of both somatic and germline sex determination as well as in the process of dosage compensation (Fig. 2) (Baker, 1989; Cline, 1993).

The structure of *Sex-lethal* is complex, with 10 exons and two promoter regions dispersed over a region of 25 kb (Samuels *et al.,* 1991) (Fig. 3). The use of the different promoters, different exons, and different polyadenylation sites leads to the production of at least 10 different RNA species, with varying patterns of expression. Three transcripts are specific to the male (4.3, 3.3, and 2.1 kb) and four to the female (4.1, two transcripts of 3.1, and 1.9 kb). One of the 3.1 kb transcripts and the 1.9-kb transcript are probably germline-dependent, since their levels of expression are reduced in abdomens that contain no ovaries. In addition, the initial activation of *Sex-lethal* results in the production of early transcripts in the female embryo (Fig. 3). These transcripts are derived from the early promoter (P_E) in response to the X:A signal (X:A = 1). Alternate splicing and the use of different polyadenylation signals give rise to three transcripts of 3.7, 2.6, and 1.6 kb. *In situ* hybridisation to whole-mount embryos indicates that these early transcripts are present in the embryo prior to pole-cell formation (Keyes *et al.,* 1992) (Fig. 4). The signal peaks in embryos at about nuclear division 12 and begins to decline until germ band extension, when it can no longer be detected. Neither early transcripts nor protein are detected in the pole cells (these are the germline primordia, Fig. 4). Not surprisingly, these transcripts are not present in flies mutant for *da.* The early proteins have been suggested to act in establishing the positive feedback loop for *Sex-lethal* autoregulation as they are present in the embryo at the time when the early functions of *Sex-lethal* occur (Salz *et al.,* 1989).

The next step in the regulation of *Sex-lethal* occurs at the level of RNA splicing. The main difference between the male transcripts and those of the female is in the incorporation of exon 3 (male-specific exon) in the male. This exon contains several translational stop codons, resulting in the formation of a truncated protein in the male. In the female, SXL protein translated from the early transcripts directs the splicing pattern of the later transcripts such that the male-specific exon is spliced out, enabling a full-length protein to be produced. These proteins maintain the productive mode of splicing.

Sequence analysis of the female cDNAs indicates that they contain a long open reading frame (ORF) which extends from exon 2 to at least exon 8 (see Fig. 3). This gives a predicted protein product of approximately 354 amino acids (Bell *et al.,* 1988; Samuels *et al.,* 1991). The sequence shows two conserved domains, RMM1 and RMM2, which show significant sequence homology to a conserved RNA binding domain found in other RNA binding proteins (RNPs) (Bell *et al.,* 1988). This family of proteins is able to bind both RNA and single-stranded DNA and functions by binding to various RNA species (including its own) to direct their

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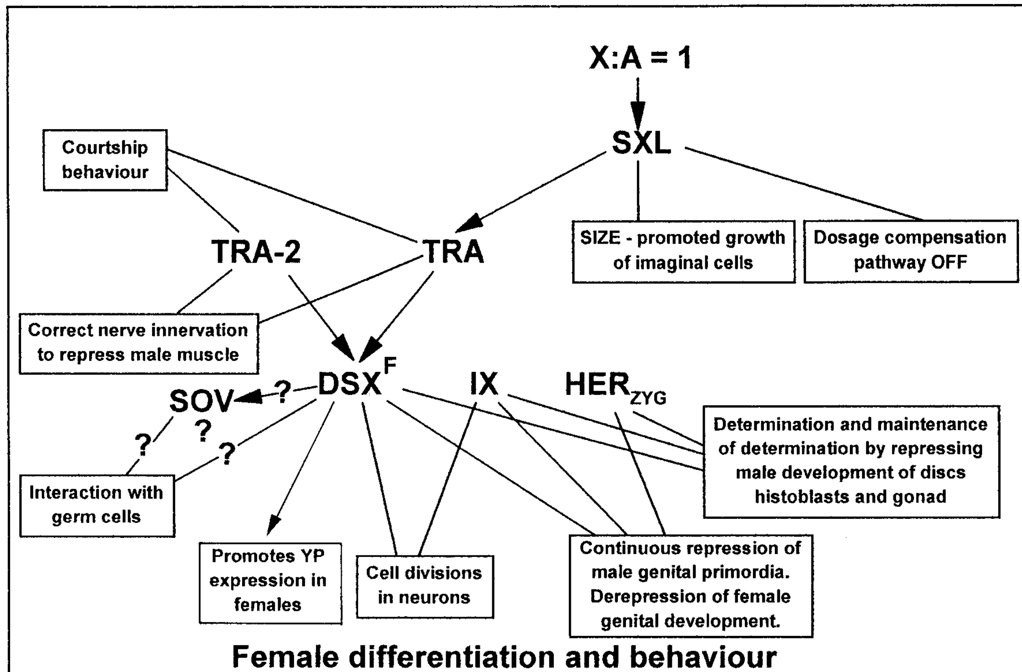

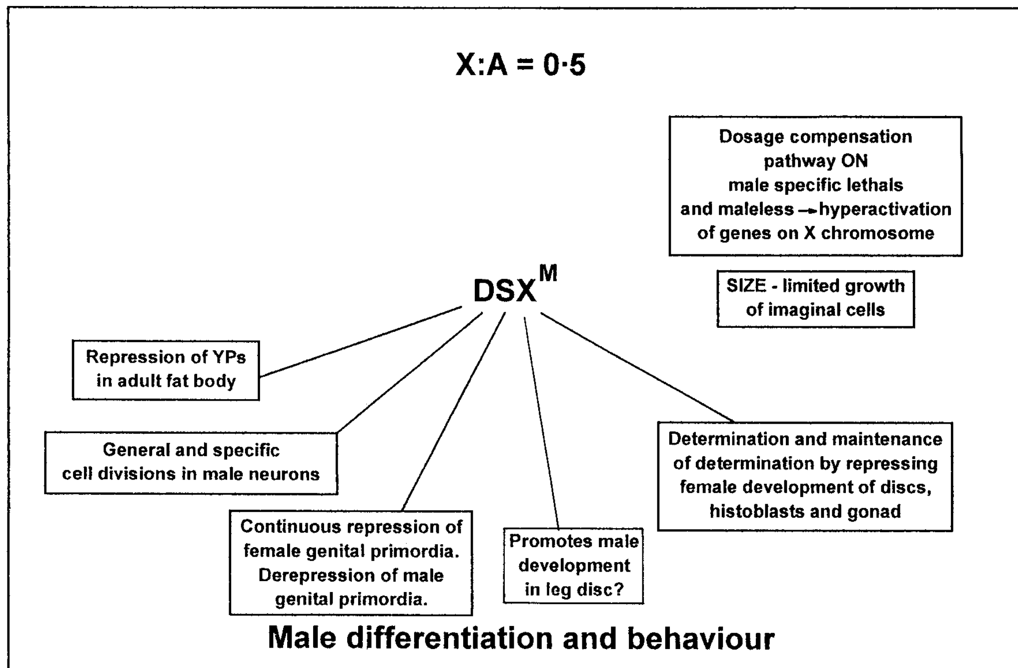

FIG. 2. Diagrams of sex determination in female and male somatic cells. X:A, X chromosome to autosome ratio; SXL, *Sex-lethal* transcript is spliced in female mode and makes a functional protein; TRA, *transformer* transcript is spliced in female mode in the presence of SXL protein and encodes a functional protein; TRA-2, *transformer-2* product is required; DSX^F, *doublesex* transcript is spliced in female mode in the presence of TRA and TRA-2 protein and makes a female-specific protein which regulates the expression of downstream genes; DSX^M, in the absence of TRA protein, *doublesex* transcript is spliced in the male mode to produce a male-specific protein that regulates the expression of downstream genes; IX, *intersex* product interacts with DSX^F to regulate downstream genes in females and affects courtship behaviour in males; HER_{ZYG}, the zygotic product of the *hermaphrodite* (*her*) gene is required for female differentiation independently of DSX. Arrows represent interactions shown experimentally. Arrows with question marks demonstrate possible interactions. *tra* does not induce germ cells but is required for differentiation of female germ cells.

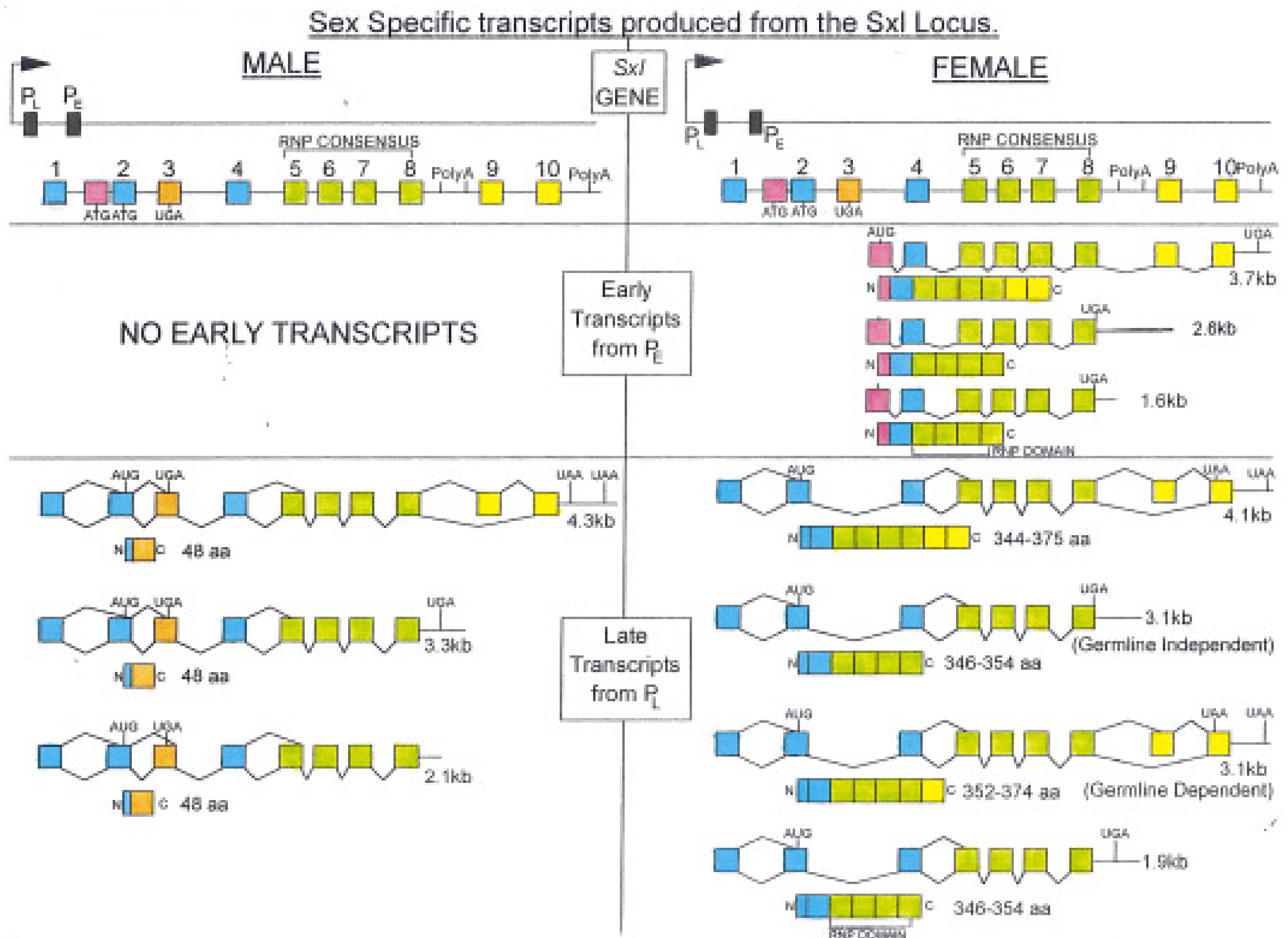

FIG. 3. The structure of the *Sex-lethal* transcription unit. The positions of the two promoters (early P_E and late P_L) are indicated. In the female, early proteins from P_E direct the splicing of the late transcripts from P_L into the female mode. Different forms of the protein are produced by the use of different splice and polyadenylation sites. These proteins all contain the ribonucleoprotein (RNP) binding domain that is required for *Sxl* function. In the male, where no early transcripts are produced, no splicing of the late transcripts occurs. Consequently the third exon (which contains stop codons) is included, producing a truncated protein that does not contain the RNP domain.

splicing pattern (Samuels *et al.*, 1994). The mechanism by which this sex-specific splicing is achieved will be discussed in the section dealing with the *tra* and *tra-2* genes.

As expected, the presence of SXL protein differs between the two sexes. It is present in female embryos and absent in male embryos (Bopp *et al.*, 1991) (see Fig. 4). In addition, the open reading frames differ among the different classes of female-specific RNAs, depending on the different splice sites and polyadenylation signals. Most of these differences occur at the carboxy terminus of the protein and all of the

FIG. 4. Developmental distribution of SXL protein. (a) In the embryo (i) SXL is found in all the syncytial nuclei except those which form the pole cells. At cellular blastoderm (ii), SXL remains localised to the nuclei. During the later stages of development, SXL can be detected in probably all somatic tissue such as the third instar larval salivary gland nuclei (iii). (b) Embryos stained using anti-SXL antibody. Antibody is stained pink and so female embryos appear pink and male embryos remain blue. No SXL protein can be detected in the pole cells (white arrow). (c) SXL protein can also be detected in male and female adults. In the female, two major species of protein (36 and 38 kDa) can be detected in all tissues. Additionally, two minor species of 40 and 42 kDa can be detected in the ovary. In the male, two smaller species of 33 and 35 kDa can be detected in the head and thorax. The functional significance of these protein varients is not known.

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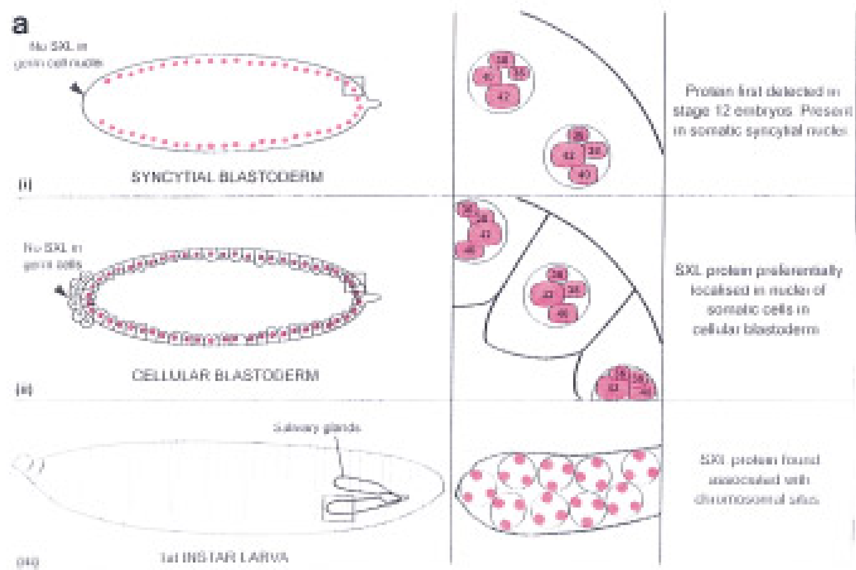
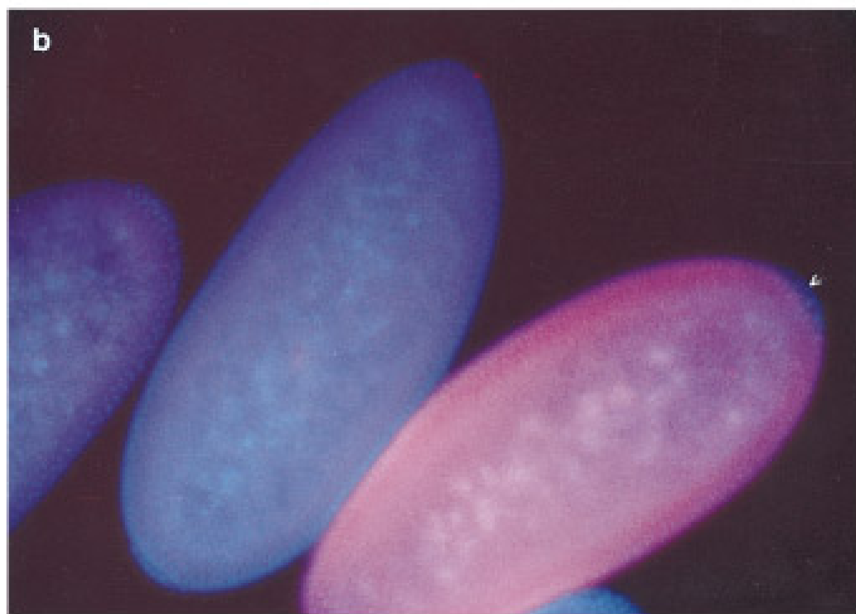
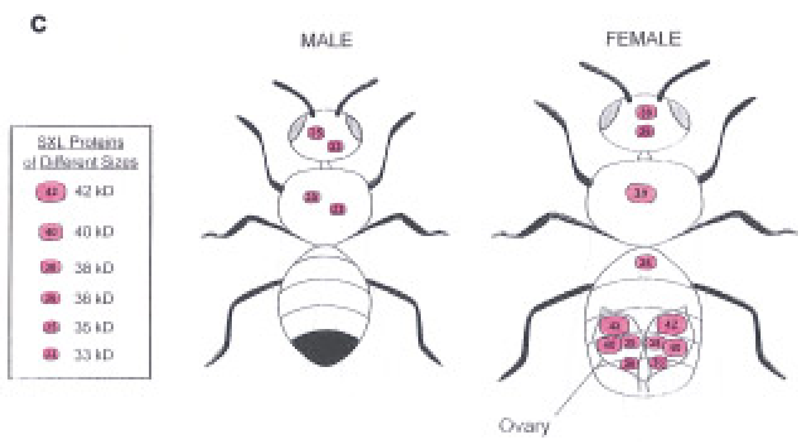

a
No SXL in germ cell nuclei
SYNCYTIAL BLASTODERM
Protein first detected in stage 12 embryos. Present in somatic syncytial nuclei
No SXL in germ cells
CELLULAR BLASTODERM
SXL protein preferentially localised in nuclei of somatic cells in cellular blastoderm
Salivary glands
1st INSTAR LARVA
SXL protein found associated with chromosomal sites


b


c
MALE
FEMALE
SXL Proteins of Different Sizes
42 kD
40 kD
38 kD
36 kD
35 kD
33 kD
Ovary

predicted female products contain both RNP domains. The distribution of the SXL protein was assessed using monoclonal antibodies raised against sequences present downstream of the male-specific exon. Two prominent proteins (38 and 36 kDa) were detected in the adult female but not in the male. These two variants derive from alternate utilisation of the 3′ splice site of exon 5. There was a differential distribution of these proteins within the fly. Both these proteins could be detected in the ovaries and head. In dissected carcasses containing no ovaries, the 38-kDa protein was the major species, with lower levels of the 36-kDa protein present. The predominant species in the thorax was the 38-kDa protein. In addition to these two species, two other minor forms of the protein (40 and 42 kDa) could be detected in ovaries and early embryos (Bopp *et al.,* 1991).

Surprisingly, SXL protein could also be detected in the adult male. Two species of 35 and 33 kDa are detected in the thorax and head; these are more prominent in the head than in the thorax and cannot be detected in the abdomen. Protein levels are 20–40 times lower than those detected in females. These proteins have been demonstrated to be derived from the *Sxl* locus, since they are absent in males carrying deletions for *Sxl.* They are similar to the low-abundance SXL proteins, which are also detected in females. It is possible that these proteins are produced from translational initiation codons downstream of exon 3. However, they do not appear to function in splicing (Bopp *et al.,* 1991), since no female-like *Sxl* or *tra* transcripts are detected in males.

SXL protein has been shown to be preferentially localised to the nucleus. In the interphase nucleus during embryogenesis, SXL protein can be detected as regions of intense staining superimposed on more diffusely staining nucleoplasm, suggesting that high levels of SXL protein have been accumulated (Bopp *et al.,* 1991).

Sex-lethal and Germline Sex Determination

In contrast to the process of sex determination in the soma, the mechanism by which sex determination occurs in the germline is still unclear. The known factors are shown in Fig. 5. As described previously, the *Sex-lethal* early transcripts (and protein) are not present in the germline primordia (Bopp *et al.,* 1991; Keyes *et al.,* 1992). In addition, the numerator elements *sis-a, sis-b,* and *runt* have been shown by pole-cell transplantation experiments not to be essential for *Sxl* activation in the germline. Pole cells from embryos mutant for all three of these loci have been shown to produce functional germ cells when transplanted to a wild-type background (Granadino *et al.,* 1993; Steinmann-Zwicky, 1994). In contrast to the somatic process, the genes *tra*, *tra-2*, and *dsx* have no function in female germline development (Marsh and Wieschaus, 1978; Schüpbach, 1982). *tra-2,* however, is required in males for the production of motile sperm (Fuller, 1993). Germ cells that are 1X:2A (i.e., male) attempt to develop as spermatocytes irrespective of the sex of the surrounding somatic tissue. In a female somatic background, these germ cells assume an intersexual identity. *Sxl* activation in the germline, in common with the process in the soma, is cell autonomous and is essential for female development. However, 2X:2A germ cells (i.e., female) also require an inductive signal to start and/or complete sexual development, which depends upon the phenotypic sex of the surrounding soma. Consequently, intersexual cells are produced in a male somatic background while oocytes develop in a female background. Any spermatocytes which are produced arrest in the primary spermatocyte stage. This suggests (at least in the female) that inductive interactions are required between the soma and the germline in order to direct the correct sex determination of the germ cells (Nöthiger *et al.,* 1989; Steinmann-Zwicky, 1992). This signal is established during embryogenesis after somatic sex determination has occurred and therefore the correct expression of the downstream sex-determining genes is required. The inductive interactions may be mediated through the action of *tra-2.* XX flies mutant for both *dsx* and *tra* express significant levels of female *Sxl* activity. In *tra-2* mutant XX individuals, *Sxl* is expressed in the male mode. Since *tra-2* is not expressed sex-specifically, it has been postulated that it interacts with an unknown gene (which may be under the control of *Sxl*) to control the inductive signal from the soma to the germline (D. Bopp, personal communication). This inductive signal has been postulated to act in one of two ways: either 2X germ cells undergo male development unless they receive an inductive signal from the female soma resulting in *Sxl* activation, thus promoting female development; or 2X germ cells undergo oogenesis unless they receive an inducing signal from the male soma to repress *Sxl,* allowing male development to occur.

Several loci have been isolated which appear to function during germline sex determination. Mutations in the genes *snf* (Salz, 1992), *fl(2)d* (Granadino *et al.,* 1990), and some alleles of the *ovarian tumour* (*otu*) locus (Pauli *et al.,* 1993) result in the formation of multicellular cysts in the ovary. This phenotype is similar to that observed with several *Sxl* germline-specific mutations. SXL protein is absent in the ovaries of flies mutant for *snf* or otu^{onc} genes (Bopp *et al.,* 1993). As described previously, the *snf* locus encodes a protein which exhibits homology to the U1A snRNP protein. It is therefore likely that the SNF protein acts to establish the correct splicing of *Sxl* transcripts in both the soma and the germline (Bopp *et al.,* 1993; Oliver *et al.,* 1993; Flickinger and Salz, 1994). Although *otu* has been cloned and sequenced, no significant homology to other sequences has been obtained. It has been postulated that these genes may act in establishing *Sxl* autoregulation. Mutations in the gene *bag-of-marbles* (McKearin and Spradling, 1990) result in sterility in both males and females, with production of undifferentiated cysts observed in both the testis and the ovary. The germ cells in these cysts are morphologically similar to germline stem cells, gonial cells or, in some cases, spermatocytes. Ovaries from these flies have normal levels of SXL but the localisation of the protein is perturbed (Bopp *et al.,* 1993). Unlike the previous classes of mutants, mutations at the *ovo* locus are still able to produce rudimentary

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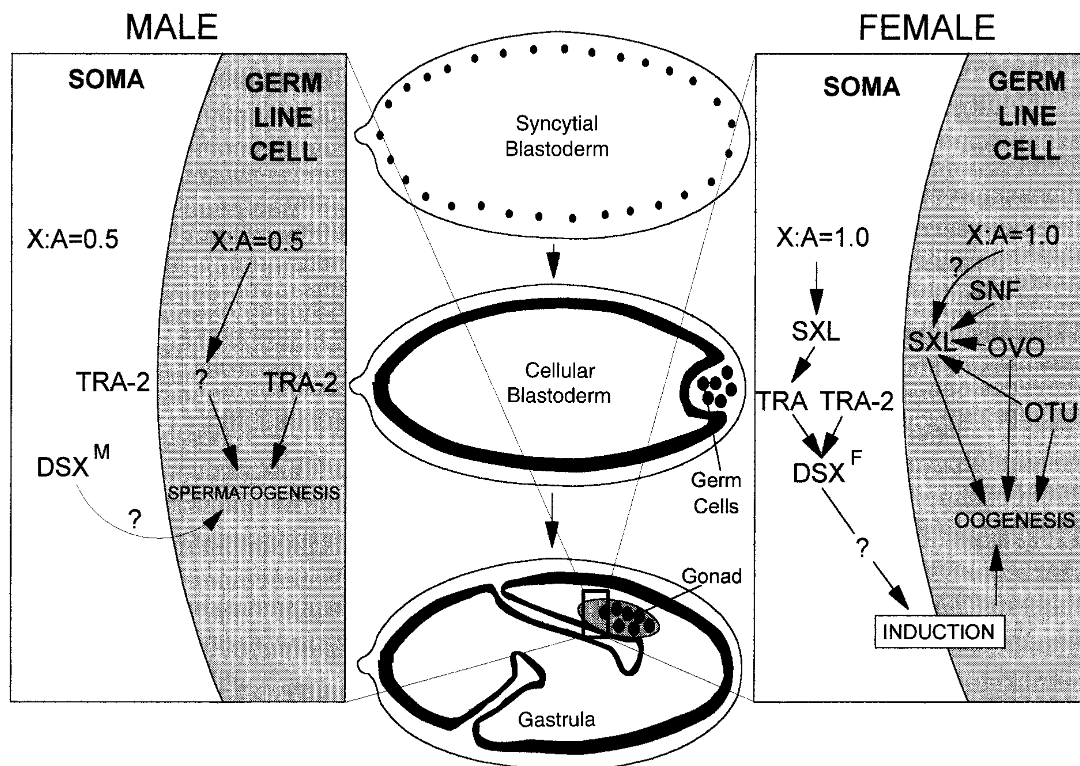

FIG. 5. A schematic representation of the process of germline sex determination. In the female, the process of germline sex determination differs from the process in the soma. Inductive interactions with the soma (mediated by the genes of the somatic sex determination hierarchy) are required, as well as a cell autonomous signal mediated by SXL. The signal from the X:A ratio activates SXL. This, in conjunction with the OVO and *ovarian tumour* (OTU) protein and the inductive interactions from the soma, determines the sex of the germline. In the male, there does not appear to be a repressive inductive interaction with the soma. Germline sex determination appears to be partly cell autonomous. DSX^F, *doublesex* female-specific protein; DSX^M, *doublesex* male-specific protein; OTU, *ovarian tumour* protein; OVO, *ovo* protein; SNF, *Sans fille* protein.; TRA, *transformer* protein; TRA-2, *transformer-2* protein.

germ cells. For many alleles, the process of oogenesis is defective: any egg chambers that do form degenerate and do not produce functional eggs. The *ovo* gene has been characterised at the molecular level and is found to encode a zinc finger-containing transcription factor (Oliver *et al.,* 1993). The pleiotrophic gene *fused* (*fu*) also affects germline sex determination (Bopp *et al.,* 1993; Oliver *et al.,* 1993). *fu* encodes a serine/threonine kinase and may act in soma/germline communication. *Sxl* is expressed in flies mutant for *fu* but is not correctly distributed.

Sex-lethal and Dosage Compensation

In some organisms in which one sex is heterogametic, the process of dosage compensation is essential to compensate for the functional aneuploidy that exists in the heterogametic sex. This dosage compensation can be achieved in several ways. One of the X-chromosomes may be inactivated; the 2X-chromosomes may be transcribed at a lower rate than the single X-chromosome; or the single X-chromosome may be transcribed at a faster rate than the 2X-chromosomes. In *Drosophila*, in which the male is the heterogametic sex, dosage compensation is achieved by hypertranscription of the single male X-chromosome (Mukherjee and Beermann, 1965). The incorporation of uridine into transcripts derived from the male X-chromosome relative to the autosomes was shown to be substantially higher than that of the female X-chromosome. This ensures the equalisation of levels of gene products in the male and female.

Sex-lethal and Male-Specific Lethals

Trans-acting regulators of dosage compensation have been identified. These comprise a group of four autosomal loci known collectively as the *male-specific lethals.* These are the *male-specific lethal-1* (*msl-1*), *male-specific lethal-2* (*msl-2*) (Belote and Lucchesi, 1980a), *male-specific lethal-3* (*msl-3*) (Lucchesi *et al.,* 1982), and *maleless* (*mle*) loci (Belote and Lucchesi, 1980b). As their name suggests, mutations in these genes are lethal to males but not females. It

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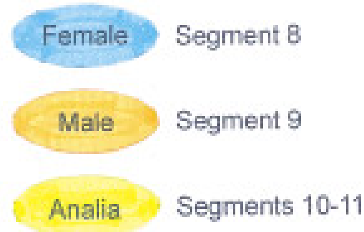
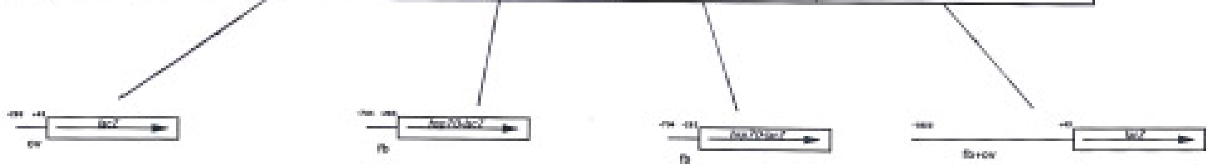


FIG. 8. The segmental origins of the imaginal disc primordia are shown. These three groups of primordial cells fuse to form the discs of the larvae and differentiate into the male and female genitalia and analia. Blue shows the derivatives of the female primordial cells, orange the male primordial cells, and yellow the analia which differentiate into different structures in male and female flies. The female primordial cells do not grow or differentiate in males and the male primordial cells do not grow or differentiate in females. They do, however, remain an integral but small population of cells within the genital disc.

	Female gonad	Fat body – female	Fat body – male	Male gonad
EMBRYO	Female gonad DSXF - expression unknown Determination of female gonad	Fat body - both sexes DSX - expression unknown Determination of primordia for adult fat body		Male gonad DSXM - expression unknown Determination of male gonad (more cells than female)
LARVA	DSXF expressed Development of female gonad			DSXM expressed Development of male gonad
PUPA	DSXF expressed Differentiation of ovary	Differentiation of female fat body	Differentiation of male fat body	DSXM expressed Differentiation of testis
ADULT	YPs expressed in follicle cells Trans acting factors unknown DSXF independent	Female DSXF expressed YPs transcribed in fat body Requires fat body factors DSXF enhances	Male DSXM expressed YPs repressed in fat body DSXM represses	YPs repressed Mechanism unknown Involvement DSX unknown



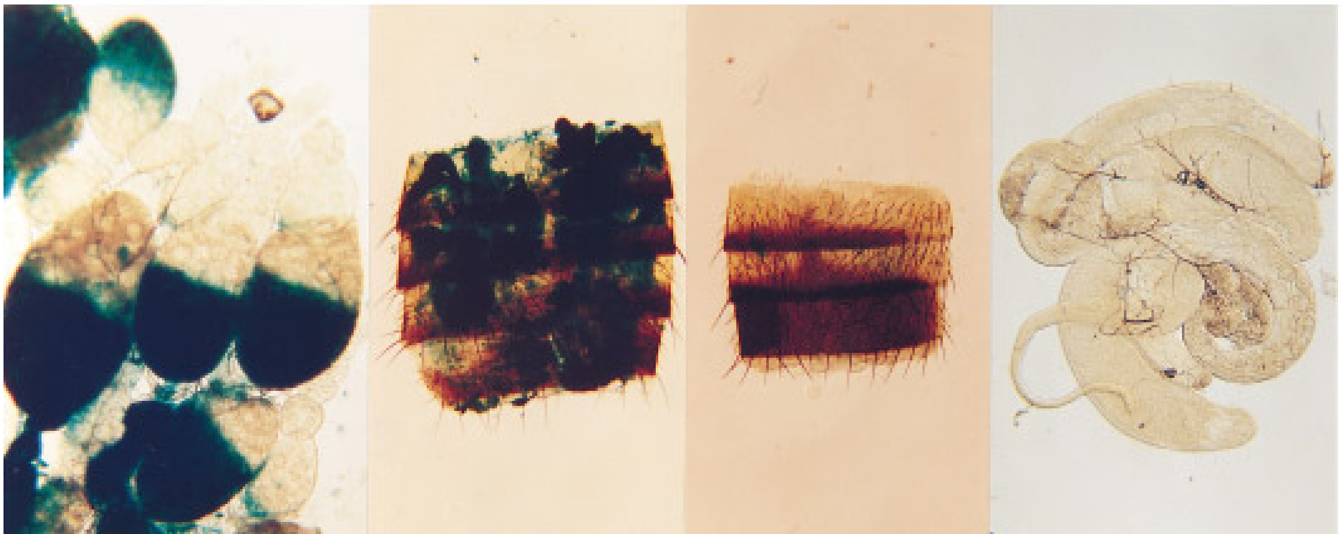


FIG. 9. The role of DSX in *yp* regulation. In the embryo DSX directs the decision as to whether the gonad will develop along a male or female developmental pathway and maintains this in the larva. In the pupa the ovary or testis differentiates and then, under the control of unknown tissue-specific factors, the *yp* genes are repressed in the testis but expressed in specific follicle cells at defined stages of oogenesis. The expression of *dsx* is unknown during fat body development. The adult fat body differentiates during metamorphosis. DSX then directs repression of the *yp* genes in the male fat body. Expression requires fat body-specific factors. Using a *lacZ* reporter the DNA sequences flanking the *yp* genes which direct this sex- and tissue-specific expression have been identified. Examples are shown for *yp3*. A fragment from −285 to +49 bp directs expression in the ovary but not the fat body. A fragment from −704 to −285 directs female fat body expression. This same construct is not expressed in the male fat body. These regions, either independently or, as shown in the figure, together, show no expression in the testis. fb, fat body; ov, ovary; hsp 70-lacZ, *heat-shock protein-70* glue promoter fused to a *lacZ* reporter gene; DSXF, *doublesex* female protein; DSXM, *doublesex* male protein.

has been postulated that these genes function in a common pathway, since mutations in one of these loci is just as detrimental as mutations in more than one. Mutations in *mle*, *msl-1*, and *msl-2* result in a 50–60% reduction in the levels of gene transcription of the X-chromosome evident in wild-type males (Baker *et al.,* 1994).

The *mle*, *msl-1*, and *msl-3* genes have been characterised at the molecular level. Proteins from these genes are expressed in both males and females; however, their pattern of distribution differs greatly between the sexes (Baker *et al.,* 1994). They are seen to associate with many sites on the male X-chromosome, but are not present on the female X-chromosome. This association leads to a significant increase in the presence of acetylated histone H_4. This observation suggests that these proteins act directly to regulate the process of dosage compensation and, in addition, that all these proteins may regulate the same set of genes. *mle*, in contrast to the rest of the *msl* proteins, is able to associate with other chromosomal locations without the interactions of the other loci. It is however unclear whether this additional binding to the chromosomes is part of dosage compensation or whether it represents another function. *mle* is allelic to *nap,* a gene which affects the activity of sodium channels (Kernan *et al.,* 1991).

Regulation by Sex-lethal

The function of *Sxl* in dosage compensation was inferred by the reciprocal male and female lethal phenotypes of gain of function and loss of function mutations. The immediate target of *Sxl* during dosage compensation is *msl-2* (Zhou *et al.,* 1995). *Sxl* functions by preventing the splicing out of a female-specific leader sequence in the *msl-2* transcripts; consequently no MSL-2 protein is produced. In the male, this sequence is spliced out, resulting in the production of functional MSL-2 protein (Zhou *et al.,* 1995). In flies which are mosaic for *Sxl* expression, MSL proteins were only associated with the X-chromosome in cells which were not expressing *Sxl*. *mle* produces multiple transcripts during development (Kuroda *et al.,* 1991), *msl-1* codes for three transcripts (Palmer *et al.,* 1994), and *msl-3* has at least three transcripts (Baker *et al.,* 1994). All of these appear to be equivalent in both males and females, showing that the regulation of these transcripts by *Sxl* is not direct. The *msl-2* 4-kb transcript can also be detected in both males and females. However, it appears that this transcript is more abundant in males than in females. Also, the female transcript appears to be slightly larger than that of the male. As described previously, this is due to the presence of an untranslated leader sequence found in the female transcript but spliced out in males. The predicted MSL-2 protein contains both a RING finger motif and a metallothionein-like domain. This protein is absent from females. MSL-2 protein has also been demonstrated to bind to the X-chromosome at the same sites as MLE and MSL-1. As discussed previously, regulation of *msl-2* may be mediated by the female-specific leader sequence. The 5′ and 3′ ends of this intron contain stretches of thymine residues similar to those found in the *Sxl* consensus binding site (Zhou *et al.,* 1995).

In polytene chromosome squashes the male X-chromosome is more open and diffuse than that of the female. It has been proposed that this altered chromatin configuration is important in allowing hypertranscription to occur. Sequence analysis of the *mle* locus has shown that it exhibits similarity with both RNA helicase A and the DEAH RNA helicases (Kuroda *et al.,* 1991). It has been proposed that *mle* forms a complex with the other *msl* proteins (similar to the spliceosome complex), facilitating hypertranscription by either increasing the rate of elongation of transcripts or removing RNA from the transcription start sites.

Other mechanisms of dosage compensation have been proposed to exist. It appears that the proteins from the *Sxl* early promoter (which, as discussed earlier, directs the autosplicing of *Sxl* transcripts) may function in directing early stages of dosage compensation which are not regulated by the *msl* loci (Gergen and Wieschaus, 1986; Bernstein and Cline, 1994). Females which are homozygous for all four *msl* loci and a null allele of *Sxl* still die. If this lethality was due to inappropriate activation of the *msl* loci, then it would be expected that the additional mutations in the *msl* loci would suppress this lethality by preventing hypertranscription. This suggests that *Sxl* may act on loci other than the known *msl*s to direct dosage compensation. The X-linked gene *runt* is an example of a gene which appears to be regulated by the action of *Sxl* and not the *msl*s. Therefore loci may exist which are also regulated by *Sxl* and act on a different set of genes. It is therefore interesting to note that alleles of *Sxl* exist that affect dosage compensation later in development which are not suppressed by mutations in *mle* or *msl-1*. As described previously, it has been suggested that all four *msl* act on the same targets. Consequently it is possible that other dosage compensation loci exist which act on a different set of target genes.

Autoregulation of Sxl

A number of uridine runs have been identified in the introns both upstream and downstream of the *Sxl* male-specific exon (exon 3) and have been implicated in SXL regulation of the female-specific splicing event (Sakamoto *et al.,* 1992; Horabin and Schedl, 1993a,b; Samuels *et al.,* 1994; Wang and Bell, 1994). Sakamoto *et al.* (1992) used a cell culture transient expression system to show that deletion of several of these U-rich motifs disrupts SXL regulation. It was observed that regulation could be restored by replacing the deleted sequences with synthetic oligonucleotides. In the first real binding study of SXL, Samuels *et al.* (1994) used gel-shifts, footprinting, and UV cross-linking with purified SXL protein to demonstrate directly that SXL binds to poly-(U) runs in RNA. Both these studies suggested that SXL may bind cooperatively to adjacent poly-(U) motifs and cooperativity has been subsequently demonstrated by Wang and Bell (1994). This study showed that SXL protein binds to many sites around the male-specific exon and that,

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when bound, the proteins interact cooperatively via their N-termini. An increase in cooperativity was observed when longer RNA molecules with multiple U-rich motifs were used as a binding substrate. The authors suggest that the N-terminus of SXL may interact directly with other splicing regulatory proteins. In support of this, the hnRNP A1 has an N-terminus with a similar amino acid constituency to SXL and interacts with the splicing factor SF2/ASF.

Progress has recently been made in the elucidation of the type of mechanism used by SXL to prevent the inclusion of exon 3 in the processed female transcript. Horabin and Schedl (1993a,b) have used germline transformation to introduce altered *Sxl* minigene constructs into flies. Using RT-PCR to analyse the spliced RNA products of these mutant minigenes, they were able to ask very specific questions about what sequences around the male-specific exon are actually required for SXL regulation. Their findings have further demonstrated the importance of the poly-(U) runs both upstream and downstream of exon 3. Interestingly, it was shown that deletion of the five poly-(U) runs in the downstream intron disrupted SXL regulation much more drastically than deletion of the U runs in the upstream intron. This suggests that the critical step in preventing the inclusion of exon 3 is the blockage of the downstream intron's 5′ splice site. This is supported by the observation that when the exon 3 5′ splice site is deleted, all product in both males and females is spliced in the female mode. In summary, it appears that SXL protein prevents the inclusion of exon 3 by acting at a number of U-rich motifs which lie in the introns surrounding the exon. Exactly how this blocking occurs remains to be seen but may involve either direct modulation of splicing regulators by SXL proteins or a "nonspecific" sequestering of the whole area facilitated by SXL cooperative binding.

Sxl Regulation of *tra* Splicing

As well as modulating the splicing of its own transcript, SXL protein also regulates the splicing of the primary transcript from the gene transformer (*tra*). Cloning and characterisation of the *tra* gene has shown that, in female flies only, the choice of a downstream splice acceptor site prevents the inclusion of a translational stop codon. This facilitates the production of the active 211-aa TRA protein (Butler *et al.,* 1986; McKeown *et al.,* 1987, 1988; Boggs *et al.,* 1987—see Fig. 6). The observation that the area around the non-sex-specific splice site of the *tra* gene contains a uridine octamer sequence originally suggested that *tra* may be directly under the control of SXL. Transformation experiments have subsequently shown that SXL is responsible for the sex-specific splicing of *tra* nascent RNA and that the splice acceptor site containing this uridine octamer is required for this regulation (Sosnowski *et al.,* 1989). This was shown by introducing various constructs containing *tra* genomic DNA (transcribed by the hsp70 promoter) into a *tra*$^-$ background via germline transformation. Deletion of the non-sex-specific splice site led to a degree of *Sxl*-independent feminisation of the male, as would be expected if the function of SXL in the female is to prevent the use of this site. Deletion of the sex-specific splice site resulted in accumulation of unspliced RNA in females, a lack of female-specific RNA, and an inability of this construct to either rescue *tra*$^-$ females or transform males.

SXL blocks the non-sex-specific splice site by antagonising the essential splicing factor U2AF which binds to the same U-rich sequences as the SXL protein. SXL, however, lacks the arginine-serine "RS" repeat which is present in U2AF (Zamore *et al.,* 1992; Zhang *et al.,* 1992) as well as other splicing factors such as SF2/ASF (Ge *et al.,* 1991; Krainer *et al.,* 1991), SC35 (Fu and Maniatis, 1992), SRp20, SRp75 (Zahler *et al.,* 1992), and suppressor of white apricot (*Su*(*W*^{a}); Chou *et al.,* 1987). The 70K U1 snRNP also contains these repeats (Theissen *et al.,* 1986; Spritz *et al.,* 1987). If the RS motif is introduced into the SXL protein, it becomes constitutively active as a splicing factor, causing splicing from the same splice site which it normally blocks (Valcárcel *et al.,* 1993).

The predicted protein product from the female-specific *tra* transcript also contains an RS motif (Boggs *et al.,* 1987), indicating that the function of TRA may be to modulate splicing in the female. Su(W^a) protein can be rendered nonfunctional by deletion of its RS motif and when this is replaced with the TRA RS motif this function is restored, indicating that this motif may play a similar role in both proteins (Li and Bingham, 1991). Deletion of the Su(W^a) RS motif seemed to affect the nuclear localisation of the protein, which may suggest a possible role for this region. However, the U2AF^{65} RS motif was also shown to be essential for its *in vitro* splicing activity (Zamore *et al.,* 1992), suggesting that this motif may have more than one function.

Regulation of *tra-2* Splicing

The cloning of transformer-2 (*tra-2*) has revealed that it encodes four transcripts which are alternately spliced (Amrein *et al.,* 1988; Goralski *et al.,* 1989; Mattox *et al.,* 1990; Amrein *et al.,* 1990), as shown in Fig. 7. These transcripts potentially encode proteins with a common C terminus containing both the RS motif and the 80- to 90-amino-acid RNP motif, which is also found in SF2/ASF, SC35, SRp20, SRp75, and U2AF^{65}, as well as in the U1A and U2B″ snRNPs (reviewed in Kenan *et al.,* 1991), indicating that *tra-2* may also encode a splicing regulator.

tra-2 Function in the Male Germline

TRA-2 protein is required in the male germline, as shown by the fact that nonfunctional sperm are produced in flies mutant for this gene (Belote and Baker, 1983). Indeed, most abundant expression of *tra-2* is seen in this tissue where two male germline-specific transcripts are produced. In wild-type flies, the splicing of the M1 intron (shown in Fig. 7) is normally an inefficient process such that the concentration of the M1-containing transcript is higher than the

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(a)

U2AF ?

AUG UGA UAA

1 2 3 4 5 6 7 8 9 10 *Sxl* Primary RNA

SXL

AUG UAA

1 2 4 5 6 7 8 9 10 *Sxl* Processed RNA

SXL SXL SXL

AUG UAG UGA

1 SXL U2AF 3 *tra* Primary RNA

AUG UGA

1 2 3 *tra* Processed RNA

TRA TRA-2

AUG UGA UAG

1 2 3 4 5 6 *dsx* Primary RNA

TRA TRA TRA

AUG UGA

1 2 3 4 *dsx* Processed RNA

N DSX^F C

TRA-2 TRA-2 TRA-2

COURTSHIP
REPRESSION OF MUSCLE

EXUPERANTIA

(b)

AUG UGA UAA

1 2 3 4 5 6 7 8 9 10 *Sxl* Primary RNA

AUG UGA UAA

1 2 3 4 5 6 7 8 9 10 *Sxl* Processed RNA

AUG UAG UGA

1 2 3 *tra* Primary RNA

AUG UAG UGA

1 2 3 *tra* Processed RNA

U2AF

AUG UGA UAG

1 2 3 4 5 6 *dsx* Primary RNA

AUG UAG

1 2 3 5 6 *dsx* Processed RNA

N DSX^M C

TRA-2 TRA-2 TRA-2

SPERMATOGENESIS

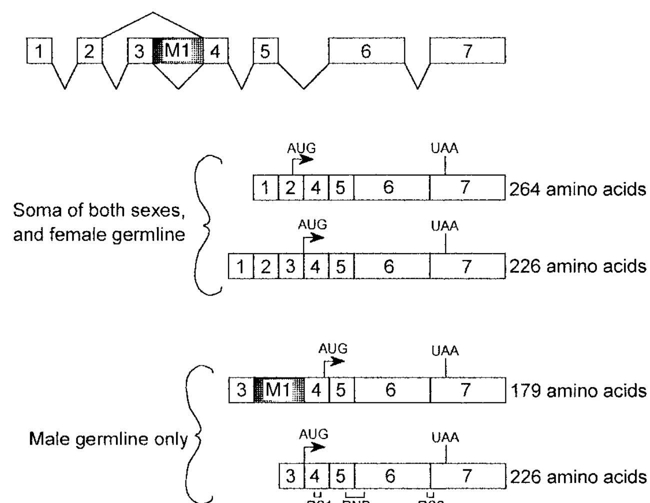

FIG. 7. Schematic diagram showing the alternate transcripts produced from the *transformer-2* locus. Exons are represented by open boxes. Removal of the M1 intron (shaded box) is thought to be inhibited by *tra-2* itself in the male germline. Tissue and sex specificity, as well as sizes of predicted proteins, are indicated for each transcript. RNP, ribonucleoprotein domain consensus sequence; RS1/RS2, arginine–serine-rich motifs.

completely spliced transcript. Analysis of the amounts of M1-containing transcript in *tra-2*⁻ flies indicates that active *tra-2* product autoregulates this transcript, preventing the splicing out of the M1 intron which occurs to completion in the *tra-2* mutant flies (Mattox and Baker, 1991). This is supported by the observation that mutation of the M1 splice sites increases the levels of M1-containing transcripts. However, ectopic expression of M1-containing cDNA is not sufficient to produce mature sperm. Thus it may be that the repression of M1 splicing by active TRA-2 acts as a type of negative feedback mechanism to regulate the levels of TRA-2 in the male germline (Mattox and Baker, 1991).

tra-2 Regulates the Splicing of *exu*

It has been shown that one function of *tra-2* in the male germline is to regulate the production of male-specific transcripts from the gene *exuperantia* (*exu*). The *exu* gene has functions in both the male and the female germline (Hazelrigg *et al.*, 1990). In females, *exu* has a maternal effect, regulating the localisation of the *bicoid* gene product in the oocyte. In males, *exu* is required in the germline for correct spermatogenesis, since mutation of the gene results in male sterility. Two sex-specific and germline-dependent *exu* transcripts have been identified; a 2.9-kb transcript which is male germline-specific and a 2.1-kb transcript which is female germline-specific. These transcripts appear to be initiated from different promoters and exhibit sex-specific processing of the 3′ UTR region (Hazelrigg and Tu, 1994). In *tra-2* mutants' production of the *exu* male-specific transcript is much less efficient, although not totally abolished. Male-specific 3′ UTR sequences are important for spermatogenic *exu* function since deletions in this region result in male sterility.

Somatic Functions of *tra-2*

Although *tra-2* is clearly important for regulating male germline sexual differentiation, no somatic function has been demonstrated for *tra-2* in the male. In the female, however, *tra-2* acts in concert with *tra* to direct most aspects of female-specific differentiation (see Slee and Bownes, 1990, for review). Mutant alleles of *tra* or *tra-2* result in transformation of females into pseudomales exhibiting male characteristics. These include male pigmentation, cuticular structures, and rudimentary testes. They are, however, of female size and are infertile due to nonfunctional sperm. Mutations in the *tra* gene have no effect on males, and *tra-2* mutants show no male somatic transformations. Null alleles of the gene *dsx* result in intersexuality of both males and females, with the phenotype seeming to result from an expression of the genes responsible for both

FIG. 6. Schematic representation showing alternate splicing of *Sxl, tra,* and *dsx* transcripts. Exons are indicated by boxes, with open reading frames shaded. Proteins involved in splicing regulation and proteins produced from the processed transcripts are shown. Although only U2AF is shown as mediating normal splicing, in fact it is only one of a number of proteins that form part of the active spliceosome. (a) In females, SXL acts at poly(U) runs in the introns 5′ and 3′ of exon 3 to prevent inclusion of this exon in the processed mRNA. This results in production of a processed *Sxl* transcript capable of translating full-length SXL protein. SXL also blocks the acceptor site at the 5′ end of exon 2 in the *tra* unprocessed RNA. This results in an alternative downstream site being used instead. Thus, a *tra* processed mRNA is produced which can give rise to active TRA protein. TRA and TRA-2 proteins act together to stabilise the splicing apparatus at the acceptor site of exon 4 in the *dsx* unprocessed RNA. In this way, a female-specific *dsx*-processed RNA is produced which gives rise to female-specific DSX^F protein. Other functions of TRA and TRA-2 are also indicated. (b) Early SXL protein is absent in males, enabling inclusion of exon 3 in the *Sxl*-processed mRNA. This *Sxl* mRNA is incapable of producing full-length SXL protein. In the absence of SXL, the acceptor site at the 5′ end of exon 2 in the *tra* unprocessed RNA can be used. This yields a processed *tra* RNA which is incapable of producing active TRA protein. In the absence of both TRA and TRA-2 proteins together, the acceptor site at the 5′ end of *dsx* exon 4 is not used and a male-specific *dsx*-processed RNA is produced. This transcript gives rise to DSX^M male-specific protein. The functions of TRA-2 in male spermatogenesis are indicated. DSX^F, *doublesex* female-specific protein; DSX^M, *doublesex* male-specific protein; SXL, *sex-lethal* protein; TRA, *transformer* protein; TRA-2, *transformer-2* protein; U2AF, splicing factor U2AF.

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male-specific and female-specific development at the individual cell level.

The epistatic relationship among *tra*, *tra-2*, and *dsx* was first shown by the construction of flies which carried double homozygous mutations in various combinations (Baker and Ridge, 1980). The epistatic gene could then be identified by virtue of its phenotype being manifest in the fly. This showed that *dsx* is epistatic to *tra*. In another set of experiments ectopic *tra* expression was shown to be unable to cause development along the female pathway in flies mutant for *tra-2* and *dsx*. In addition, molecular evidence shows that *tra* and *tra-2* are required for production of female-specific *dsx* transcripts (Nagoshi *et al.*, 1988). The above evidence shows that *dsx* is epistatic to *tra* and *tra-2* and that *tra* and *tra-2* are required for *dsx* to be expressed in the female mode, while the male functions of *dsx* are independent of *tra* and *tra-2*.

Regulation of dsx Splicing

The genetic evidence suggests that *dsx* is differentially active in both males and females, acting primarily to repress genes required for differentiation of the opposite sex. Analysis of the *dsx* transcripts showed how *tra* and *tra-2* enable this to occur (Baker and Wolfner, 1988; Burtis and Baker, 1989). Examination of cDNAs representing the 3.9-kb (male-specific) and 3.5-kb (female-specific) *dsx* transcripts shows that these messages are differentially spliced and polyadenylated but are both capable of producing large, functional proteins with sex-specific carboxyl termini. Hence it would be quite feasible for there to be differential activity in both sexes.

Germline transformants containing the female-specific *tra* cDNA fused to the hsp70 promoter have their male soma transformed to female soma. This transformation is correlated with the production of the female-specific *dsx* transcripts in the male soma, while tra^-, XX flies produce only male *dsx* mRNA (McKeown *et al.*, 1988). *tra-2* is also required for production of the female-specific *dsx* transcripts but not for production of male-specific *dsx* transcripts (Nagoshi *et al.*, 1988). Tissue culture cotransfection experiments (Hoshijima *et al.*, 1991; Ryner and Baker, 1991) allow the effects of TRA and TRA-2 proteins upon *dsx* pre-mRNA to be assessed directly by analysing the spliced products of *dsx* pre-mRNA in the presence or absence of TRA and TRA-2. These studies show that TRA and TRA-2 act in concert to positively promote the usage of the female-specific splice acceptor site, as shown in Fig. 6.

A region lying just downstream of the exon 4 acceptor site has been implicated as being involved in *tra* and *tra-2* regulation of the *dsx* pre-mRNA (Nagoshi and Baker, 1990). Lying in this region are six 13-nt repeats (*dsx* repeat element *dsx*RE), the deletion of which results in a loss of female-specific product in the cotransfection system described above (Hoshijima *et al.*, 1991; Ryner and Baker, 1991). It has been shown that TRA, TRA-2, and some SR proteins bind to the *dsx*RE *in vitro* (as shown in Fig. 6), with TRA-2 binding being dependent upon a purine rich enhancer (PRE) element present within the *dsx*RE (Hedley and Maniatis, 1991; Lynch and Maniatis, 1995). The same studies showed that the *dsx*RE and PRE elements act synergistically. Also, substitution of some of the noncanonical purines present in the polypyrimidine stretch of the female-specific acceptor site causes female-specific splicing independent of TRA and TRA-2 (Hoshijima *et al.*, 1991). This indicates that this splice site is not used in males because of its nonstandard polypyrimidine stretch. In females, TRA and TRA-2 act to stabilise the splicing apparatus at this site and thus promote its use, i.e., default splicing occurs in males, while in females regulation of female-specific splicing occurs by TRA and TRA-2 promoting the use of the nonpreferred site. Indeed, it has been demonstrated that TRA and TRA-2 work by attracting general splicing factors, including some SR proteins, to the *dsx*RE region, enabling it to function as a splicing enhancer (Tian and Maniatis, 1993).

Sequences homologous to the *dsx*RE 13-nt repeats have been identified in both *exu* and *tra-2* transcripts. Since, in the male germline, alternative processing of both these transcripts is under the control of *tra-2*, it would be expected that mutations of the repeat sequences would result in loss of *tra-2* regulation. However, mutational analysis of some of these sites has not as yet been able to establish a role for them in sex-specific splicing regulation (W. Mattox and T. Hazelrigg, personal communication).

The RNP motif of TRA-2 has been shown to be essential for its somatic and male germline functions, although it is required but not sufficient to direct RNA binding *in vitro* (Amrein *et al.*, 1994). Experiments using the yeast dihybrid assay have shown that TRA and TRA-2 physically interact with themselves, with each other, and with the general splicing factor SF2. These three proteins have been shown to be sufficient to cause *dsx* primary transcripts to be spliced in a female-specific manner. One of the TRA-2 RS motifs, RS2, is essential for *in vivo* function and for interactions in the dihybrid assay. The RS1 RS motif of TRA-2 is not essential but, if deleted, results in temperature-sensitive mutation *in vivo* and decreased sensitivity of dihybrid interactions *in vitro* and so may act as a stabiliser of protein–protein interaction (Amrein *et al.*, 1994).

In addition to promoting the usage of certain splice sites, TRA-2 also appears to be able to prevent the usage of splice sites. This is shown by the fact that *tra-2* downregulates the removal of the M1 intron from *tra-2* primary transcripts in the male germline (Mattox and Baker, 1991). This may be a result of the proximity of the splice site relative to the TRA-2 binding site, which may cause bound TRA-2 to interfere stearically with the splicing apparatus. This repressive function may alternatively be due to additional tissue-specific factors which modulate TRA-2 function.

Additional Functions of tra

For most aspects of somatic sexual differentiation, *dsx* is the last member in the hierarchy of regulatory genes. This

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view, however, in which the only sex determination function of *tra* and *tra-2* is to direct the *dsx* primary transcript to be spliced in the female mode, needs to be revised. Fresh evidence indicates that *tra* and *tra-2* also govern *dsx*-independent pathways of sex-specific differentiation.

Male flies have a sex-specific pair of muscles known as the muscle of Lawrence (MOL) (Lawrence and Johnston, 1984). These muscles span the fifth abdominal segment and were initially thought to be involved in the curling of the male abdomen during copulation. More recently, however, it has been found that flies lacking the MOL can still copulate (Gailey *et al.,* 1991). XX flies carrying null mutations of *tra* or *tra-2* develop as pseudomales which have this muscle present (Taylor, 1992). However, this effect of the *tra* and *tra-2* alleles cannot be due to their function in *dsx* regulation, as the muscle is present in XY individuals mutant for *dsx* but is absent in XX *dsx* mutant flies. Thus, the repressive function that active TRA and TRA-2 proteins exert on the development of this muscle must act via a pathway that is independent of *dsx*. Transplantation of nuclei between males and females has shown that the identity of this muscle is not autonomous but depends upon the sex of the innervating axons (Lawrence and Johnston, 1986).

Recent studies on courtship behaviour have also pointed towards the presence of at least one branch of regulatory genes which are governed by *tra* and *tra-2* but not by *dsx*. In wild-type flies, mating involves a number of male-specific courtship behaviours which are readily observable (Spieth, 1974; Ehrman, 1978). Courtship is initiated with the male tapping the female with his forelegs, orienting toward her, and following her. He then begins a courtship song by extending one wing and vibrating it. This is followed by the male extending his proboscis and licking the female's genitalia and finally copulation is attempted. Females are largely sedentary during mating, although reception and rejection behaviours are observed.

Temperature shift experiments using a temperature-sensitive allele of *tra-2* have shown that absence of male behaviour in the female is dependent upon activity of the *tra-2* gene, such that inactivation of this gene in females from the late larval stage onwards results in appearance of male courtship elements (Belote and Baker, 1987). Flies of the genotype XX;dsx^- exhibit no male courtship behaviour suggesting that DSX^F does not normally act to repress male courtship behaviour in the female. Similarly XX;dsx^D flies which constitutively express DSX^M do not attempt to court despite the fact that they are male in morphology. This indicates that DSX^M does not activate male courtship in the male (Taylor *et al.,* 1994). These experiments argue that there is no role for *dsx* in regulating courtship behaviour. However, it has been observed that XY;dsx^- flies court much less than normal males. These flies also elicit more courtship than wild-type adult males, even though such flies do not produce characteristically female pheromones (McRobert and Tompkins, 1985; Jallon *et al.,* 1988). At first glance, this would indicate a direct role for *dsx* in regulating this behaviour. Taylor *et al.* (1994) observed, however, that although a large proportion of XY;dsx^- flies did not court at all, at least one fly carrying each tested allele exhibited male courtship behaviours up to and including wing extension. The lack of attempted copulation of XY;dsx^- flies may be explained by their morphology, which makes this a physical impossibility. This led Taylor *et al.* (1994) to propose that the anomalous expression of both male- and female-specific genes in dsx^- flies, due to lack of the repressive function of both DSX^M and DSX^F proteins, may lead to developmental abnormalities, resulting in the XY;dsx^- fly being less able to sense attractive females. Thus, even though the neural identity of the fly remains male, it would be less likely to court. This may also account for the increased sex appeal of these flies. Young wild-type males show both a lower courtship frequency and a higher elicitation of courtship than do wild-type adult males. The developmental burden caused by expression of both male- and female-specific genes in the XY;dsx^- flies may result in a retardation of maturation which causes these flies to retain their sex appeal and low courtship frequency after the time when wild-type males would have lost theirs. Evidence that the CNS of XY;dsx^- flies is essentially male comes from analysis of the courtship song which, although not exactly wild type, is still clearly recognisable. This is in marked contrast to the anomalous song produced by gynandromorphs when much of the thoracic nervous system is diplo-X (Taylor *et al.,* 1994).

Taken together, the above evidence suggests that *tra* and *tra-2* regulate the sexual identity of the fly regarding courtship behaviour, but *dsx* does not appear to be required, implying a branch of regulatory genes under the control of *tra* and *tra-2* but not *dsx*.

A candidate gene for being involved in such a pathway is the *fruitless* (*fru*) gene, which has marked effects on male courtship behaviour (Gailey *et al.,* 1991; Taylor *et al.,* 1994). Males with extreme *fru* alleles court vigorously but do not attempt copulation and are unable to curl their abdomen. Defects are also observed in the courtship song of these mutants. With regard to the possibility that this gene may be involved in a pathway of regulation governed by *tra* and *tra-2* but not *dsx*, two aspects of the *fru* phenotype are particularly interesting. First, certain alleles result in the absence of the MOL, although this does not account for the inability of these flies to curl their abdomen since weaker *fru* alleles which lack this muscle are still able to copulate. As discussed above, *tra* and *tra-2* are required to prevent the formation of this muscle in the female, while *dsx* and *ix* are not. However, whether *tra* and *tra-2* play any part in *fru* regulation is unknown. Second, severe *fru* alleles result in male flies courting nonspecifically such that males are courted with equal vigour as females. Interestingly, lack of courtship discrimination by male flies has also resulted from ectopically expressing *tra* in the antennal lobes or mushroom bodies (Ferveur *et al.,* 1995; O'Dell *et al.,* 1995). Again, although mutation of *fru* and missexpression of *tra* have similar effects with regard to loss of courtship discrim-

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ination in the male, it is not known whether these two genes form part of a common pathway.

The localised expression of *tra* described above was achieved using a *tra* cDNA under the control of the yeast Upstream Activator Sequence (UAS). This construct was introduced into the genome via P-element-mediated germline transformation. To express *tra* in a specific tissue, it is necessary to produce the yeast UAS-activator protein GAL4 in that tissue. This was done by transforming embryos with a P-element construct containing a GAL4 cDNA under the control of a weak promoter. This weak promoter requires the "help" of a tissue-specific enhancer to express significant levels of GAL4. Thus, the tissue localisation of GAL4 protein in these "enhancer-trap" strains depends entirely upon which tissue-specific enhancers the P-element construct comes under the control. By selecting strains which express GAL4 brain specifically and crossing them to the UAS–*tra* strain, brain-specific expression of *tra* can be achieved.

It is becoming increasingly clear that what was previously thought to be a linear hierarchy of regulatory genes is in fact a branched pathway, with sex differentiation genes lying directly under the control of *tra* and *tra-2* as well as *dsx*. It is likely that there are a number of different branches at the level of *tra* and *tra-2* and the elucidation of these processes will no doubt be the basis of future work.

The Control of Male and Female Sexual Differentiation by dsx

The major morphological differences between males and females are apparent during differentiation of the adult at metamorphosis. Many genes expressed uniquely in adult male or female somatic cells have been identified. These include the components of the vitelline membrane and chorion in females (e.g., Waring and Mahowald, 1979; Fargnoli and Waring, 1982; Kafatos *et al.,* 1985) and components of the accessory gland in males (e.g., Schäfer, 1986; Chen *et al.,* 1988; Monsma and Wolfner, 1988; DeBenedetto *et al.,* 1990). Yet most of the genes encoding these sex-specific proteins are not directly controlled by the *dsx* gene in the adult. All of the products mentioned above are made in sexually unique tissues or organs and the regulation of the genes encoding them depends upon the presence or absence of tissue-specific factors. The *dsx* gene has played its role earlier in development by determining and maintaining the state of determination throughout embryonic and larval growth and is no longer required once the cells differentiate. The exceptions to this are the *yolk protein* (*yp*) genes expressed in the female (but not the male) adult fat body (Bownes and Nöthiger, 1981) and the *glucose dehydrogenase* gene expressed in a specific pattern in the male and female reproductive tract (Feng *et al.,* 1991). Both these examples are of genes expressed in a tissue found in adults of both sexes, but with some unique sexually dimorphic functions, showing that *dsx* can function after differentiation to control sex-specific gene expression in certain tissues.

The major developmental decisions executed by *dsx* therefore occur during embryonic and larval development. Its functions are well documented by genetic and developmental studies, such as the analysis of mosaics and gynandromorph flies which contain cells of each sex within the same organism (Schüpbach *et al.,* 1978). The role of *dsx* is perhaps best illustrated for the genital disc, which gives rise to the adult analia and genitalia and comprises three distinct groups of primordial cells. One group will eventually differentiate into either male or female analia depending upon whether DSX^F or DSX^M is expressed. There is, however, a selection between the other two groups of cells, with one group growing in the female and the other in the male and differentiating into the very different genitalia of the two sexes. This means that *dsx* is able to select either the repression of or promote the growth of whole primordia as well as cause a single group of cells to select between two alternate developmental pathways (Epper and Nöthiger, 1982). How this is achieved is not clear but presumably depends upon the position of the primordial cells in the fly and upon interaction with the segment polarity and/or the segment identifying homeotic genes. Figure 8 shows the development of the male and female genital primordia, discs, and structures generated at metamorphosis in males and females.

Analysis of mosaic patches of sexually transformed cells and temperature shift experiments with a temperature-sensitive allele of *transformer-2* (*tra-2*^{ts}) shows that *dsx* is required throughout the growth of genital discs, functioning not only to set the cells along a specific developmental pathway, but also to maintain that determined state throughout the subsequent cell divisions. Thus *dsx* is required to maintain the appropriate sexual determination of cells (Wieschaus and Nöthiger, 1982; Epper and Bryant, 1983). Consistent with these results is the observation that *dsx* mutant flies differentiate both male and female pattern elements, indicating that both groups of primordial cells develop to some extent when neither DSX^F or DSX^M protein is present (Nöthiger *et al.,* 1987).

The main activity of DSX^F and DSX^M seems, from genetic studies, to be to repress the expression of genes needed in the other sex. There are, however, some indications that the proteins may also promote sex-specific gene expression. There are sex-specific differences in the cell divisions which generate the abdominal neurons of the adult. Once the female neuroblasts stop dividing, the terminal abdominal neuroblasts of the male undergo extra divisions. In the absence of *dsx* (in contrast to the genitalia) the cells do not divide at all in either sex. Thus *dsx* is required for the non-sex-specific cell divisions prior to the sexually dimorphic cell divisions (Taylor and Truman, 1992).

The male foreleg carries the sex comb and the pattern of neuronal axons differs between the male and female first legs. Recent experiments using ectopic expression of a *dsx* male-specific cDNA in flies showed that sex comb morphol-

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ogy could be induced not only in female forelegs but also on second and third legs of both sexes. This has been used to indicate that DSX^M positively promotes the development of male-specific structures of the foreleg (Jursnich and Burtis, 1993). However, this should be viewed with care, since *dsx* nulls do not have a similar phenotype and the *dsx*^{D} mutants, which constitutively express the male DSX protein, do not have sex combs on other legs. It is possible that *dsx* is not normally expressed at all in these cells and high levels of expression may lead to new phenotypes by interactions with new combinations of tissue-specific transcription factors.

One interesting point to note is that a lack of DSX proteins, or an expression of both forms of DSX^F and DSX^M in the same fly, leads to a similar phenotype, namely intersexual flies. When no DSX protein is present, presumably the sex-differentiation genes of both sexes are derepressed and thus both sets of genes are expressed. When both gene products are present it is possible that they interfere with each other's function, again leading to a partial derepression of the sex-differentiation genes.

Although *dsx* is not involved in sex determination within germ cells, its correct expression is essential in somatic cells of the gonad for germ cells to be able to develop along a female developmental pathway (Steinmann-Zwicky, 1992). Thus there is signalling between somatic cells and germ cells during development of the germline and gonad to ensure compatibility between germ cell and somatic cell sex differentiation (see section on *Sex-lethal* and germline sex determination).

Whilst we know a great deal at the level of genetic decisions about developmental fate control by the alternately spliced products of the *dsx* gene, we have no idea what the target genes of DSX^F and DSX^M are within imaginal primordia and histoblasts during development. In fact the only immediately identified downstream targets of *dsx* are the female-specific *yolk protein* genes expressed in the adult fat body. The *glucose dehydrogenase* gene expressed in the reproductive tract may also be a target but this has not been investigated at the molecular level.

For normal female differentiation to occur, two other genes are required. These are *ix* (Baker and Belote, 1983) and *her*, both of which have zygotic functions essential for normal female differentiation, yet act downstream and independently of the normal sex-specific splicing of *dsx* (Pultz *et al.,* 1994; Pultz and Baker, 1995). It seems likely that these gene products interact with DSX^F to bring about female-specific differentiation. The molecular nature of *ix* remains to be elucidated, but it is clear that *dsx* alone does not generate the developmental decisions during embryonic and larval development.

Execution of the Signal: Interactions of dsx with Downstream Sex Differentiation Genes

Despite the wealth of information about the developmental consequences of mutations in *dsx* we have information only about its molecular interactions with one family of downstream genes, encoding the female-specific yolk proteins. Even with these genes we really don't understand the nature of sexual selection, since DSX^F and DSX^M bind to the same DNA fragment and give the same footprint, showing that they contact the same DNA bases (Burtis *et al.,* 1991), yet *in vivo* this leads to *yolk protein* gene repression in males and expression in females.

The three *yolk protein* genes (*yp1*, *yp2*, and *yp3*) are expressed in the follicle cells of the ovary and in the adult female fat body (reviewed in Bownes, 1994). Their expression in the follicle cells is not directly controlled by *dsx* but is cell-type-specific once the *dsx*-dependent decision to develop and differentiate an ovary rather than a testis has been made (Bownes *et al.,* 1990). In the fat body, *yp* gene expression is directly controlled by the sex-determination hierarchy (Belote *et al.,* 1985). XX flies with mutations in *tra-2* or *dsx*, for example, can have their *yp* genes repressed and XY flies with mutations at *dsx* can express the *yp* genes in the fat body. This latter finding suggests that the main function of DSX^M is to repress expression in males. There is some evidence that DSX^F promotes *yp* gene expression in females (Coschigano and Wensink, 1993).

A number of *cis*-acting DNA sequences have been identified which confer female fat body expression of *yp1*, *yp2*, and *yp3*, using a variety of reporter gene systems (Garabedian *et al.,* 1985, 1986; Logan *et al.,* 1989; Logan and Wensink, 1990; Liddell and Bownes, 1991; Abrahamsen *et al.,* 1993; Ronaldson and Bownes, 1995). One of these, a 125-bp fragment located 5′ of *yp1*, called the fat body enhancer (FBE), has been extensively studied, but it should be noted that it is not the only region flanking *yp1* and *yp2* that can direct female fat body expression of these two divergently transcribed genes. DSX protein, which contains a zinc finger DNA binding domain, binds four times within the FBE. DSX^F and DSX^M differ at their carboxy termini, not in the DNA binding region, and can compete for the same DNA binding sites in the FBE (Burtis *et al.,* 1991; Erdman and Burtis, 1993).

A number of other proteins, fat body transacting factors, have been shown *in vitro* to bind to the FBE (Abel *et al.,* 1992: Falb and Maniatis, 1992). These include both enhancers and repressors, the transcriptional activator box B-binding factor-2, the CCAAT/enhancer binding protein, and the adult enhancer factor 1. These proteins regulate *alcohol dehydrogenase* gene expression in the fat body and give footprints which overlap with those of DSX in the FBE.

It seems that the interaction with these other trans-acting factors may differ according to whether DSX^F or DSX^M is present. When neither is present transcription will proceed. When DSX^F alone is present, transcription also proceeds, but when DSX^M alone binds, transcription is repressed. Perhaps DSX^M is less easily displaced by transcriptional activators in the fat body. The presence of both DSX^F and DSX^M allows transcription of the *yp* genes, possibly because they interfere with each other's function.

To understand how this is achieved will require a combi-

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nation of *in vitro* gel shift and footprint assays to investigate which combinations of factors will bind or be displaced by each other. Mutations induced in the binding sequences will then be essential to see if these *in vitro* studies are valid by analysis of the *in vivo* function of the altered binding sites. Other, as yet unidentified, gene products, such as those encoded by *ix* (Baker and Belote, 1983), may also have critical roles to play in the sex specificity of *yp* gene expression. Figure 9 shows our current understanding of the role of *dsx* in *yp* gene expression.

Ovarian development requires interaction between the germ cells and somatic cells. We know that signals sent from the sexually determined female somatic cells to the germ cells are essential for female germline development. A gene which is downstream of *dsx* in the sex determination pathway and which is essential for ovarian and female germline development is *small ovaries (Wayne et al.,* 1995). This mutation maps to the X-chromosome and once cloned would be a good possibility for an additional target for *dsx* gene regulation.

CONCLUSIONS

Alternate splicing provides a mechanism for generating families of related proteins with either dramatic or subtly different functions, depending upon the system. In *Drosophila* this has been exploited to regulate sexual determination and differentiation by a genetic cascade of alternately spliced products. The alternate splicing of the transcripts encoding both splicing factors and DNA binding proteins is quite well understood, but there are a number of branches in the pathway that remain to be elucidated, along with understanding the mechanism by which the differentially spliced products of the DNA binding proteins can bring about sex-specific transcription of target genes.

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