Genetic and developmental analysis of the sex-determining gene 'double sex' (*dsx*) of *Drosophila melanogaster*

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Summary

Sex determination in *Drosophila* depends on the ratio of X chromosomes to sets of autosomes $(X:A)$. This chromosomal signal is used to regulate a few control genes whose state of activity selects either the male or the female sexual pathway. We have studied the structure and function of *dsx* (double sex) which appears to be the last regulatory gene on whose function the sexual pathway eventually depends. We have mutagenized the locus, varied the doses of dominant *dsx*-mutations and wildtype alleles, and combined different *dsx*-alleles with recessive mutations in other sex-determining genes, such as *ix*, *tra-2* and *tra*.

The locus *dsx* harbours two genetic functions, dsx^m to implement the male program, dsx^f to implement the female program. We found that dsx^m and dsx^f can mutate independently although most mutations abolish both functions. We conclude that dsx^m and dsx^f each have their specific domain, but also share a large region of DNA that is essential for both functions. We present evidence that the dominant mutations correspond to a constitutive expression of the male-determining function dsx^m, with the simultaneous abolishment of the female-determining function dsx^f. This effect can be counteracted by two doses of expressed dsx^f so that a female phenotype results. The products of one dose of expressed dsx^m and one dose of expressed dsx^f in the same cell appear to neutralize each other which leads to a null phenotype. The mutant combinations suggest that the product of dsx^f requires the products of ix^+, $tra-2^+$ and tra^+ to become functional.

Introduction

In *Drosophila*, the ratio of X chromosomes to sets of autosomes $(X:A)$ is the only discriminator between male (XYAA) and female (XXAA) development (Bridges, 1921). All processes related to sex, i.e. dosage compensation and sex determination in the soma and in the germ line, depend on the $X:A$ ratio. This quantitative signal, however, does not directly control the sex differentiation genes, but uses a small number of regulatory genes whose state of activity then selects either the male or the female pathway. Seven genes are known so far that govern sexual differentiation of somatic cells, namely *da*, *sis-a*, *Sxl*, *ix*, *tra-2*, *tra* and *dsx*. Of these, *dsx* (double sex), described by Hildreth (1965), occupies a special position: Mutant combinations suggest that it lies at the end of a regulatory pathway and that the functional state of *dsx* actually determines the sexual phenotype of somatic cells (Baker & Ridge, 1980). Mutations at *dsx* can be recessive or dominant, and they can affect both chromosomal sexes, or only XY, or only XX. The mutations cause an intersexual phenotype at the cellular level as if the male and the female programs were simultaneously expressed in the same cell. The *dsx*-locus is also special since it seems to harbour two functions, one required for male development and one for female development (for review see Baker & Belote, 1983; Nöthiger & Steinmann-Zwicky, 1985; for *sis-a* see Cline, 1986).

In an attempt to learn more about structure, function and regulation of *dsx*, we undertook a genetic and developmental analysis of this locus and studied the phenotypic effects of various mutant alleles and genetic combinations.

2. Materials and Methods

Unless noted otherwise, all crosses were done at 25 °C. The flies were reared on standard food (corn meal, sugar, yeast, agar). For genetic symbols see Lindsley & Grell (1968). The mutation dsx^D was described by

Duncan & Kaufman (1975), dsx^{Mas} by Nöthiger *et al.* (1980); dsx^T is another dominant allele of *dsx*, found in W. Gehring's laboratory in Basel.

2.1. *Screening of mutants at the dsx-locus*

Males with the genotype $X/Y.B^s$; *mwh jv* p^p were fed with EMS (Lewis & Bacher, 1968), and massmated to *Basc*; *Sb/TM3, Ser* females. Progeny was raised at room temperature. Male parents were discarded after five days to prevent clustering of new mutations. Single F_1 males of the genotype *Basc/Y.B^s*; *mwh jv* $p^p/TM3$, *Ser* and single females of the genotype $+/$ *Basc*: *mwh jv* $p^p/TM3$, *Ser* were crossed with two *th st tra cp Df(dsx) bx sr e^s/TM3, Sb Ser* flies. (The *Df(dsx)* was recovered as a 'revertant' of dsx^D and identified as a deficiency described as *Df(3R)*, dsx^{D+R5} in Duncan & Kaufman, 1975.)

The single crosses were set up at 29 °C in an attempt to isolate also temperature-sensitivie mutants. In the F_2, new *dsx*-mutants as well as lethal factors were uncovered by the chromosome carrying the deficiency *Df(dsx)*, and could be kept over the *TM3* chromosome as a balanced stock. Flies expressing a new *dsx* mutation were mounted in Faure's solution and studied under a compound microscope.

2.2. *Complementation analysis*

Crosses were performed between new *dsx*-alleles *inter se* and also with previously existing mutants. The resulting flies were checked for fertility and afterwards mounted for microscopical inspection of their sexually dimorphic structures. Recessive lethals which are located within *Df(dsx)* were tested for complementation; they were also combined with a viable *dsx*-allele to see whether any of the lethals displayed a *dsx* phenotype.

2.3. *Induction of reversions of two dominant dsx mutations, dsx^D and dsx^T*

(a) dsx^D *Sb e/TM6* males were treated with EMS (Lewis & Bacher, 1968) and crossed with *y w* females. The progeny was screened for normal and fertile *Sb* females.

(b) Males with the genotype $X/Y.B^s$; $dsx^T/TM3$, *Sb Ser e* were irradiated with 4000 r (Philips MG160, 2 mm Al filter, 25 cm distance, 150 kV, 14 mA, 8 min) and crossed with *e* females. Sb^+ Ser^+ e^+ daughters showing a normal female phenotype indicated a dsx^T revertant. Revertants were testcrossed and analysed over different *dsx*-alleles and over *Df(dsx)*.

2.4. *Dosage effects*

The effect of dsx^D in the presence of two doses of dsx^+ was studied in (1) triploid females (3X;3A), (2) triploid intersexes (2X;3A) and (3) normal diploids (2X;2A). For this purpose, animals with the genotypes (1) $\widehat{XX}/X;dsx^D/+/+$, (2) $XX;dsx^D/+/+$, and (3) $\widehat{XX}/Y.dsx^+$; $dsx^D/+$ were constructed and their sexually dimorphic structures were compared with those of X/X; $dsx^D/+$ flies. The $Y.dsx^+$ is a translocation of the region 84D10, 11; 85A1-3 to the Y chromosome; besides dsx^+, it also carries p^+ and $Rg(pbx)$, and is described as $T(Y;3)P92$ in Duncan & Kaufman (1975).

Table 1. *Scheme for quantifying the sexual phenotype of flies in abdominal segments A7–A9*

| Abdominal segments with values assigned to the structures of wild-type males and females | | | | | | | | |
| A7 (7 tergite/sternite) | | | A8 (Female genitalia) | | | A9 (Male genitalia) | | |
Structure	v^a	w^c	Structure	v	w	Structure	v	w
T7[b]	0·7		VP	0·56		CL	0·12	
S7	0·3	3	T8	0·34	3	SP	0·04	
			SPT	0·1		LP	0·14	4
						GA	0·38	
						HY	0·14	
						PE	0·18	

[a] A value (*v*) was ascribed to each structure so that the sum of these values added up to 1·0 for the imaginal derivatives of one abdominal segment in a normal wild-type female or male. In the intersexes, the structures were most of the times smaller and incomplete and sometimes absent, yielding a sum considerably below 1·0 (see Fig. 1).

[b] Abbreviations: T7, S7, tergite 7, sternite 7; VP, vaginal plate; T8, tergite 8; SPT, spermatheca; CL, clasper; SP, sperm pump; LP, lateral plate; GA, genital arch; HY, hypandrium; PE, penis with parameres.

[c] For the calculation of the 'sex index' (see Fig. 2), the values of the three segments were given a weight (*w*) roughly proportional to the size of the imaginal structures in normal flies. Thus, for each genotype the lengths of the shaded columns in Fig. 1 were multiplied by 3 for A7 and A8, and by 4 for A9; the three values obtained were then added and divided by 10 (3+3+4). In this way, a value between 0·0 (completely male) and 1·0 (completely female) was obtained (for explanation see Fig. 1). This value places an intersex on a line between a male and a female (see Fig. 2). For illustration of the structures see Fig. 3.
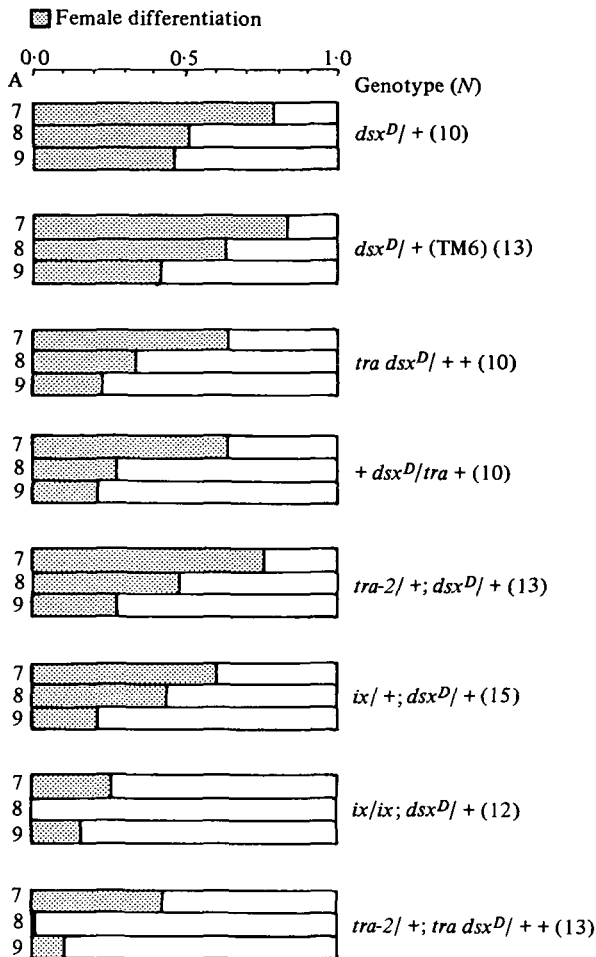
☑ Female differentiation

Fig. 1. Characterization of the sexual phenotype produced by combinations of dsx^D with recessive mutations in other sex-determining genes. The dotted area represents the degree of femaleness, calculated as described in section 2.6 of Materials and Methods and in Table 1. Abdominal segments 7, 8 and 9 (A7, A8, A9) display strong sexual differences: A7 produces tergite and sternite 7 only in females, A8 the female genitalia, and A9 the male genitalia, respectively. A reduction in size and number of female structures in A7 and A8 represents a male tendency, a reduction in the inventory of male structures in A9 signals a female tendency. Thus, for A7 and A8 the shaded area gives a measure for the number and size of female structures differentiated by a given genotype; for A9, however, the shaded area, i.e. degree of femaleness, derives from incompleteness of male structures. A short shaded column in A7 and A8 means that we observed relatively few and small *female* structures, in A9 it means that there were many and large *male* structures present. The combined values for A7, A8, A9 were used to calculate a 'sex index' (Table 1 and Fig. 2). A, Abdominal segments 7–9; *N*, number of analysed flies. For complete genotypes see Materials and Methods.

2.5. *Ovary transplantation*

After we had found that $\widehat{XX}/Y.dsx^+$; $dsx^D/+$ were sterile females with well developed ovaries, we tested whether such ovaries could give rise to functional eggs if transplanted into normal females (series A and B), and whether $\widehat{XX}/Y.dsx^+$; $dsx^D/+$ would become fertile if provided with normal ovaries (series C and D). Ovaries were transplanted between larvae of the late 3rd instar as described by Ursprung (1967).

2.5.1. *Series A and B*

Host larvae were obtained by crossing *Basc*; *TM3, Ser/Sb* females to $Fs(1)K1237/Y$ males. $Fs(1)K1237$ is a fully penetrant, dominant mutation that sterilizes females by blocking oogenesis before stage 4 (see King, 1970 for description of stages); the mutation acts only in the germ line (Busson *et al.* 1983). Since *K1237* host ovaries remain small, donor ovaries can develop without competition (Monod & Poulson, 1936). (The $Fs(1)K1237$ mutation was kindly provided by M. Gans, Gif-sur-Yvette.)

Donors were produced by crossing $C(1)RM$, $y\,v/Y.dsx^+$; *red/red* females to $y/Y.dsx^+$; $dsx^D\,Sb\,e/red$ males. Half of the female larvae had the genotype $C(1)RM$, $y\,v/Y.dsx^+$; $dsx^D\,Sb\,e/red$; the other half carried three doses of dsx^+ and is also listed in Table 2. After transplantation, surviving female hosts were test-crossed to either y/Y; $dsx^{Mas}/TM3$, *Sb Ser* males (series A), or to $X/Y.dsx^+$; $dsx^{Mas}/TM2$, *Ubx* males (series B). The genotype of the donor ovary, abbreviated as dsx^D and dsx^+ in the right-most column in Table 2, was inferred from the genetic markers seen in the offspring. The $C(1)RM$, $y\,v$ chromosome is abbreviated as $\widehat{XX}$ in Table 2.

2.5.2. *Series C and D*

Donor ovaries from $y\,w\,f^{36a}\,mal$ larvae were injected into $C(1)RM$, $y\,v/Y.dsx^+$; $dsx^D\,Sb\,e/red$ hosts (series C) or into $Basc/T(1;Y;3)$ *H1*; $dsx^D\,Sb\,e/+$ hosts (series D). The $T(1;Y;3)H1$ is an X chromosome that carries a duplication for $dsx^+\,p^+Rg(pbx)$ besides the markers $y\,w^a\,f$. It was constructed in our laboratory by A. Hilfiker (1983), and is abbreviated as $X.dsx^+$ in Table 2.

All surviving host females were mated with wildtype males, tested for fertility, and dissected after a few days. The donor ovaries carry the mutation *mal* that eliminates aldehyde oxidase (AO) activity and that can be used as a histochemical marker (Janning, 1976). Thus, donor and host ovaries could be distinguished and we could determine whether a donor ovary had attached to the host's gonoducts.

2.6. *Interaction of dsx-alleles with mutations at other sex-determining loci*

(a) To study the epistatic relation between *dsx* alleles and transformer (*tra*), the following double-mutants were constructed, both in X/X and X/Y animals: (1) $tra\,dsx^m/tra\,dsx^m$, (2) $tra\,dsx^f/tra\,dsx^f$, (3) $tra\,dsx^{mf}/tra\,dsx^{mf}$ and (4) $tra\,dsx^D/tra\,+$. The recombinant chromosome $tra\,dsx^D$ was obtained by irradiating X/Y; $dsx^D\,Sb\,e/th\,st\,tra\,cp\,ri\,p^p$ males with 2500 r and screening their male offspring for an
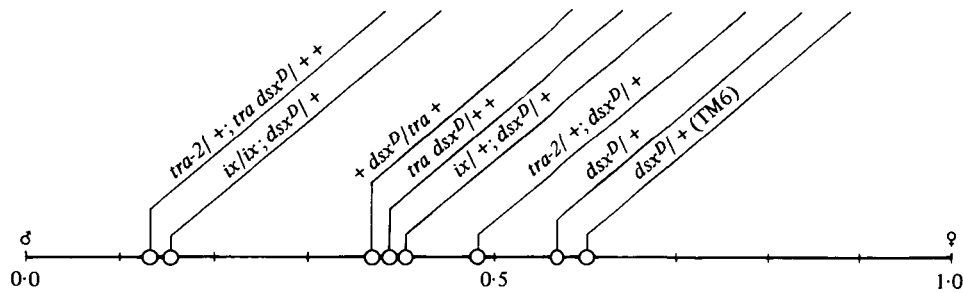
Fig. 2. Graphical representation of sexual phenotypes as characterized by a '*sex index*' between 0·0 (normal male) and 1·0 (normal female). For calculation of the 'sex index' see Table 1.

induced recombinational event between *st* and *ri* or p^p. Individual recombinant males were tested for the presence of *tra* and *dsx^D* in *cis* on the *Sb e* chromosome. (The symbol *dsx^{mf}* designates alleles that have lost both functions, *m* and *f*.)

(b) The interaction of *dsx^D* with *ix*, *tra-2* and *tra* was studied in the following six genotypes:

(1) X/X; *ix/SM5*; *dsx^D Sb e/* +,
(2) X/X; *ix/ix*; *dsx^D Sb e/* +,
(3) X/X; *tra-2/* + ; *dsx^D Sb e/* +,
(4) X/X; *tra/* + *dsx^D Sb e*,
(5) X/X; *tra-2/* + ; *tra dsx^D Sb e/* +
(6) X/X; +/*SM5*; *tra dsx^D Sb e/* +.

Two genotypes, X/X; +/*SM5*; *dsx^D Sb e/* + and X/X; *dsx^D Sb e/TM6*, served as references.

The sexually dimorphic structures of these flies were microscopically analysed. For this purpose, the flies were macerated in hot 10% NaOH, washed in H_2O, and mounted under coverslips in Faure's solution. For abdominal segment 7 and the genitalia, the sexual phenotype was quantified by estimating the size of a structure relative to the corresponding size in normal males or females (for details of the procedure see Table 1 and Janning *et al.* 1983). Thus, each intersexual fly is characterized by three values between 0 and 1 (Fig. 1): one value for segment 7, one for the female genitalia (segment 8), and one for the male genitalia (segment 9). The values are quantitative and do not indicate whether the structures were morphologically normal or abnormal.

The values for the structures of these three segments were used to calculate a 'sex index' which characterizes the intersex by assigning a place to each of the genotypes on a line between normal male and normal female (see Table 1, Fig. 2).

3. Results

3.1. *Mutagenesis*

From a total of 7218 single crosses, 4854 had progeny. Among these, eight new *dsx* alleles and 40 recessive lethals were recovered within the deficiency of *dsx^{D+R5}* (referred to as *Df(dsx)* in this paper). The *dsx* alleles fall into three categories:

(i) *dsx^{mf}*, recessive, affecting both chromosomal sexes, 6 alleles. These mutations transform both

chromosomal sexes, although with different expressivity, into intersexes of the type described by Hildreth (1965) and Baker & Ridge (1980) for the original *dsx*-allele. Our new alleles were tested in trans with Hildreth's (1965) original *dsx* allele and with *Df(dsx)*. The allele *dsx^{31}* gives the same strong intersexual phenotype as the original *dsx* allele which presumably corresponds to a loss of function (Baker & Ridge, 1980). Four mutants (*dsx^{53}*, *dsx^{501}*, *dsx^{10R}*, *dsx^{584}*) have a much stronger effect on XX than on XY animals. The latter look almost normal, but display slightly rounded analia, an incomplete penis apparatus, and are sterile. In *XX*-animals, all four mutants give a clear intersexual phenotype in combination with Hildreth's (1965) *dsx*-allele. All chromosomes carrying a new *dsx* mutation were homozygous lethal in both sexes; since they are viable over *Df(3R) dsx^{D+R5}*, they must have suffered lethal hits outside the deficiency. A sixth mutant was lost before a stock could be established.

No complementation was found among the five alleles, except for X/Y; *dsx^{584}/dsx^{501}* that were fertile males. In combination with the strong *dsx^{31}*-allele, the four weaker alleles showed a weak phenotype in *XY* and a strong phenotype in *XX*-flies. We conclude that these alleles are leaky and can still make some *dsx$^+$*-product that is at least partially active in chromosomal males.

(ii) *dsx^f*, recessive, affecting only XX animals, one allele. Animals with genotype X/X; *dsx^f/dsx^f* have a strong *dsx*-phenotype whereas X/Y; *dsx^f/dsx^f* are normal, fertile males. Transheterozygotes of the male-specific mutation *dsx^m* and our *dsx^f* yield fertile males and females indicating full complementation of the two mutations. The allele *dsx^m* was discovered and kindly provided by A. Garen and briefly described as *dsx^{136}* by Baker & Ridge (1980).

(iii) *dsx^{Dw}*, dominant, affecting only XX animals, one allele. Animals with genotype X/X;*dsx^{Dw}/* + are always sterile and show a very weak *dsx* phenotype (reduced number of vaginal teeth, cleft in dorsal anal plate). X/X; *dsx^{Dw}/dsx* show a strong *dsx*-phenotype with more male than female characteristics. *XY*-animals remain unaffected.

Lethal mutations within the dsx-deficiency. Forty lethals were detected within the interval of *Df(dsx)*. None of them gave an intersexual phenotype in

combination with a *dsx*-allele; conversely, none of the eight new *dsx* alleles was lethal over the deficiency. The complementation pattern is complex and yielded 29 different complementation groups (Leist, 1983). The lethals were not analysed further.

3.2. *Dominant mutations at the dsx-locus*

(a) *Description.* So far, three different spontaneous dominant mutations at the *dsx*-locus were recovered: dsx^D (Fung & Gowen, 1957; Duncan & Kaufman, 1975), dsx^{Mas} (Mischaikow, 1959; Nöthiger *et al.* 1980), dsx^T (kindly provided W. Gehring, Basel). They produce very similar phenotypes, and the following description thus applies to all three of them.

The terminalia of X/X; $dsx^D/+$ flies carry abnormal male and female elements, with the female genital set anterior to the male set (Fig. 3 *d*). The male genital structures are well developed, but the penis apparatus is reduced, the hypandrium mostly absent, and many bristles are abnormal. The female vaginal plate is always present, but reduced in size and with abnormal bristles. Between the vaginal plates, a mass of yellow, chitinized tissue is often enclosed, identified as a secondary rudimentary penis apparatus. The anal plates are arranged in a left–right position as in males, but the shape corresponds to a sexually intermediate form. The bristle pattern is a mosaic of male, female and intermediate bristles. The basitarsus of the foreleg carries a sex comb whose position and shape of bristles, however, are intermediate between male and female. Interestingly, the phenotype of X/X; $dsx^D/+$ is indistinguishable from that caused by homozygosity for null alleles, such as *dsx*. For a more detailed description of X/X; $dsx^D/+$ flies see Epper (1981) and Roost (1978). X/Y; $dsx^D/+$ flies are completely normal and fertile males.

X/X; dsx^D/dsx flies as well as X/X; $dsx^D/Df(dsx)$ flies are sterile pseudomales with perfect external and internal male genitalia and analia (Duncan & Kaufman, 1975; Nöthiger *et al.* 1980; Baker & Ridge, 1980). The inner genitalia are also male; the gonads are rudimentary testes and resemble those found in X/X; *tra/tra* pseudomales (Seidel, 1963).

Flies with the genotype X/X; dsx^D/dsx^f are pseudomales and indistinguishable from X/X; dsx^D/dsx.

(b) *Description of 'reversions'.* Among 2655 X/X; Sb progeny of EMS-treated X/Y; dsx^D Sb e/TM6 males, two 'reversions', designated dsx^{D+RH1} and dsx^{D+RH2}, were found. X/X; $dsx^{D+RH1}/+$ show a slight *dsx*-phenotype (reduced number of vaginal teeth, spliced dorsal anal plate), but are weakly fertile females; X/X; $dsx^{D+RH2}/+$ is a normal female. X/Y; $dsx^{D+RH1}/+$ and X/Y; $dsx^{D+RH2}/+$ are normal males.

Among 2500 X/X; *non-TM3* flies deriving from irradiated X/Y; $dsx^T/TM3$ males, one 'reversion' was found. It showed the same phenotype as dsx^{D+RH1}.

All three 'revertants' were tested in trans with dsx^m, dsx^f, dsx^{mf}, $Df(dsx)$, and dsx^D. These combinations revealed that the 'revertants' were recessive *dsx*-alleles of the type dsx^{mf}, as were the 'revertants' of dsx^D and dsx^{Mas} described by Duncan & Kaufman (1975) and by Belote *et al.* (1985). Over dsx^{mf} or $Df(dsx)$ they showed an intersexual phenotype in both chromosomal sexes; over $dsx^D Sb^+$ they transformed XX animals into pseudomales; with dsx^m or dsx^f, they gave an intersexual or normal phenotype, depending on the chromosomal sex.

3.3. *Effects of variable doses of dsx^D and dsx^+*

(a) *Sexual phenotype of triploids and diploids*

(1) *Triploid flies with the genotype* $\widehat{XX}/X$; $dsx^D/+/+$ looked like females, but were sterile, a fact already noticed by Gowen & Fung (1957). The sixth tergite was darkly pigmented; the basal row of bristles on the forelegs showed a slight *dsx*-sex comb; the outer genitalia and analia were as in normal females, except that the vaginal plates and the seventh tergite carried a reduced number of bristles; the internal genitalia were also female, but not always complete. The parovaria, the uterus and the receptaculum seminis were absent in about 1/5 of the flies studied. Ovaries were present, but they remained rudimentary in most cases; some 40 % of the animals also contained traces of testicular tissue.

(2) *Triploid intersexes with* $\widehat{XX}/Y$; $dsx^D/+/+$ displayed the general features of triploid intersexes, i.e. a mosaic pattern of male and female elements (Stern, 1966; Laugé, 1969), rather than the intersexual phenotype of *dsx* mutants. When compared to their siblings $2X$; $3A dsx^+$ that arose in the same cross, the $2X$; $3A dsx^D$ flies looked very similar. Both had typically male sex combs that contained a reduced number of bristles. The segmentation pattern was variable, with seventh and eighth tergites sometimes being present, sometimes absent. The pigmentation of the fifth and sixth tergites was a mosaic for the male and female pattern. The differences between the two genotypes were insignificant. The $2X$; $3A dsx^D$ contained fewer external male genitalia than the $2X$; $3A dsx^+$ controls, which, in view of the masculinizing action of dsx^D, is paradoxical. The effect cannot be ascribed to dsx^D, but must be the result of differences in the genetic background which is known to influence the sexual phenotype of triploid intersexes.

(3) *Diploid flies with the genotype* $\widehat{XX}/Y . dsx^+$; $dsx^D/+$ looked like normal females, but were sterile (Fig. 3 *b*). In contrast to the triploid flies with one dose of dsx^D (see above), their ovaries were well developed containing all oogenic stages up to mature eggs, and all the other internal structures appeared also normal. They mated, and few of them even laid eggs, that, however, were never fertilized. Microscopical inspection of the receptaculum seminis showed that sperm were present at 0, 0·5, 2 or 24 h after copulation, but

they were invariably immotile. In wild type control females, on the other hand, motile sperm were found at all times tested up to 24 h after copulation. It thus seems that the sperm became immobilized immediately after entry into the gonoducts of $\widehat{XX}/Y.dsx^+$; dsx^D *Sb e/red* females.

(b) Ovary transplantation

The results, summarized in Table 2, show that $\widehat{XX}/Y.dsx^+$; dsx^D *Sb e/red* ovaries, when attached to the gonoducts of a normal female, can in fact produce eggs that can be fertilized and give rise to adult flies. The experiment indicates that the gonadal soma of $\widehat{XX}/Y.dsx^+$; dsx^D *Sb e/red* is capable of supporting the development of normal eggs. The sterility focus of $\widehat{XX}/Y.dsx^+$; dsx^D *Sb e/red* females must therefore reside outside of the germ line and the gonadal soma. The fact that normal sperm is instantly inactivated in the seminal receptacle of these females suggests a focus in the gonoducts which are the derivatives of the genital disc. The situation is reminiscent of meta-females (3X;2A) which are invariably sterile and in which Schüpbach *et al.* (1978) could map the focus for sterility to the derivatives of the genital disc. The actual physiological cause for the sterility is not known, but it appears that dsx^D, in spite of two doses of dsx^+, still succeeds in partially repressing the female differentiation genes, and that the genital ducts may be particularly sensitive to this effect. The data from series C and D are consistent with this conclusion. None of the 31 $dsx^D/+/+$ host flies with an implanted *y w f mal* ovary became fertile, although at least three hosts had an attached, unstained donor ovary. The low number of attached ovaries can be explained by

the fact that the hosts in series C and D had, in contrast to those in series A and B, their own developing ovaries as competitors for attachment to the oviducts (Monod & Poulson, 1936).

(c) Flies with two dominant dsx-alleles, produced by ovary transplantation

The transplantation series A and B differ in the genotype of the tester males (Table 2) which in series B carry a Y chromosome with a duplication of dsx^+.

In series A, part of the progeny will have the genetic constitution $\widehat{XX}/Y$; dsx^D/dsx^{Mas}. Twelve such flies were produced by the three females with attached dsx^D ovaries. They were phenotypically male and sterile with rudimentary testes, resembling X/X; *tra/tra* pseudomales in every respect (Fig. 3a). This observation confirms earlier results obtained with transplanted pole cells of X/X; $dsx^D/+$ embryos (Nöthiger *et al.* 1980).

In series B, the 12 females with attached dsx^D ovaries produced an interesting genotype with two dominant *dsx*-alleles and one dose of dsx^+ ($\widehat{XX}/Y.dsx^+$; dsx^{Mas}/dsx^D). We obtained 46 animals of this genotype all of which were typical pseudomales (Fig. 3c). A microscopical inspection disclosed that the 6th sternite was evenly covered with bristles, which is a female characteristic; the 6th sternite of normal males is devoid of bristles. The analia of $\widehat{XX}/Y.dsx^+$; dsx^{Mas}/dsx^D are slightly rounded compared with those of $\widehat{XX}/Y$; dsx^{Mas}/dsx^D. The genitalia are male, but morphologically not quite normal (compare Fig. 3c with 3a). These characteristics suggest that the additional dose of dsx^+ has a very weak feminizing effect.

Table 2. *Transplantation of ovaries*

Series	Host[a]	Donor[a]	Tester male[a]	No. of surviving hosts	No. of fertile females	No. of attached donor ovaries
A	$Fs(1)K1237/Basc$; $TM3, Ser/+$	$\widehat{XX}/Y.dsx^+$; dsx^D/dsx^+ or $\widehat{XX}/Y.dsx^+$; dsx^+/dsx^+	X/Y; $dsx^{Mas}/TM3$	53	10	3 dsx^D 7 dsx^+
B	$Fs(1)K1237/Basc$; $TM3, Ser/+$	$\widehat{XX}/Y.dsx^+$; dsx^D/dsx^+ or $\widehat{XX}/Y.dsx^+$; dsx^+/dsx^+	$X/Y.dsx^+$; $dsx^{Mas}/TM2$	111	27	12 dsx^D 15 dsx^+
C	$\widehat{XX}/Y.dsx^+$; dsx^D/dsx^+	$y w f^{36a}mal$	$+/+$	8	0	1
D	$X.dsx^+/X$; dsx^D/dsx^+	$y w f^{36a}mal$	$+/+$	23	0	2

[a] For complete genotypes of hosts and donors see Materials and Methods. The genotype of the donor ovary in series A and B is abbreviated in the right-hand column as dsx^D corresponding to $\widehat{XX}/Y.dsx^+$; dsx^D/dsx^+, and as dsx^+ corresponding to $\widehat{XX}/Y.dsx^+$; dsx^+/dsx^+.
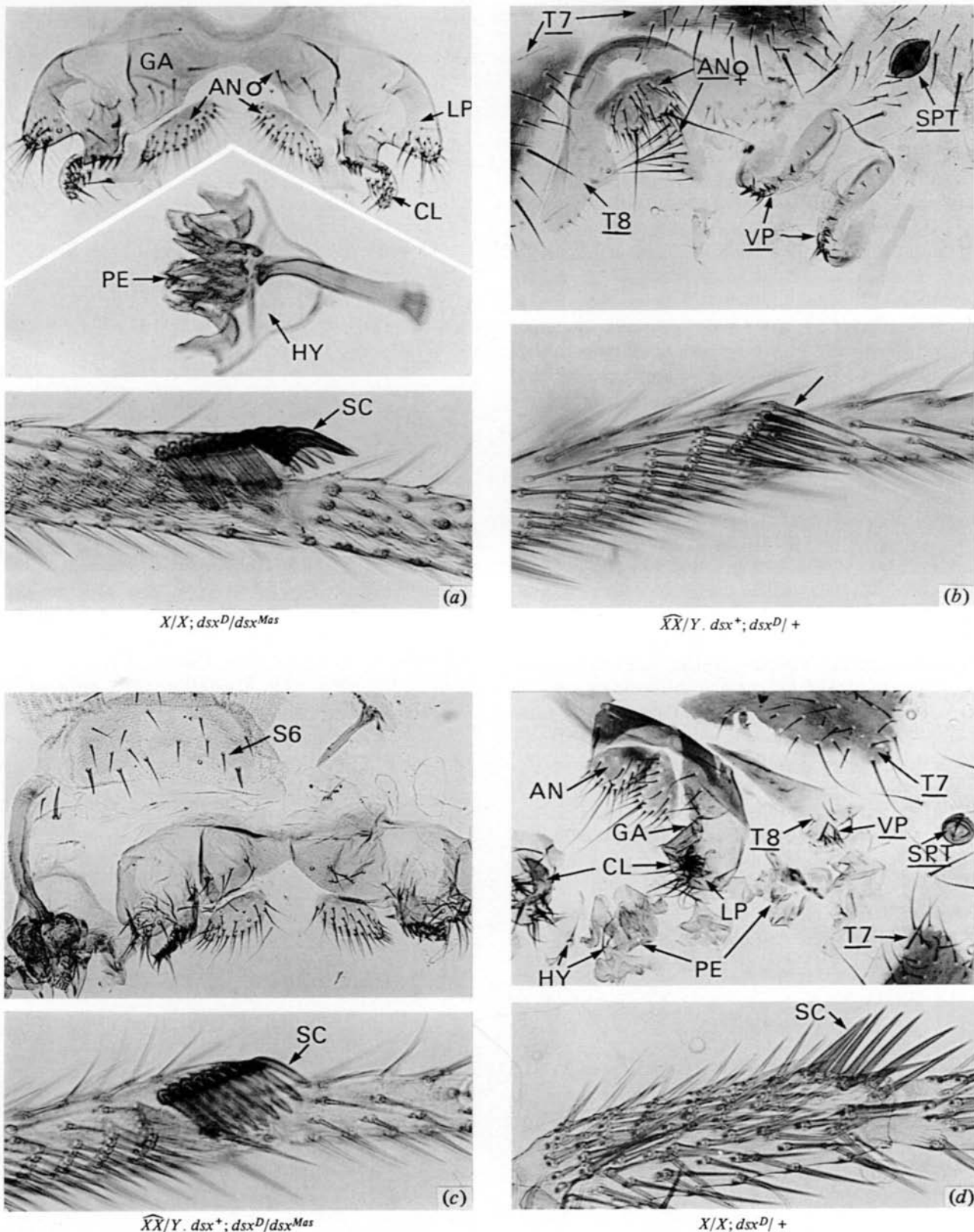
Fig. 3. Photographs of terminalia and forelegs of XX flies with variable doses of wild-type (dsx^+) and dominant mutant (dsx^D, dsx^{Mas}) alleles of *dsx*. (*a*) X/X; dsx^D/dsx^{Mas} is a normal pseudomale. (*b*) $\widehat{XX}/Y . dsx^+$; $dsx^D/+$ is practically female. Arrow points to basal row of bristles on the basitarsus of the foreleg; in males, this row is rotated by 90° and the bristles are thick with rounded tips (see SC in (*a*). (*c*) $\widehat{XX}/Y . dsx^+$; dsx^D/dsx^{Mas} is essentially pseudomale, but the male genitalia are slightly less well developed than in (*a*), the analia are more round, and sternite 6 (S6) carries bristles. (*d*) X/X; $dsx^D/+$ is typically intersexual: note intermediate shape and position of sex comb bristles (arrow SC), presence of abnormal and incomplete male and female genital structures, position, shape and bristle pattern of anal plates. Female structures (underlined): $AN♀$, anal plates; *SPT*, spermatheca; *T7*, tergite *7*; *T8*, tergite *8*; *VP*, vaginal plates. Male structures: $AN♂$, anal plates; *CL*, claspers; *GA*, genital arch; *HY*, hypandrium; *LP*, lateral plates; *PE*, penis; *SC*, sex comb.

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3.4. *Interaction of dsx-mutations with mutations at other sex-determining loci*

(a) *Homozygosity for tra.* Irrespective of their sex chromosome constitution (X/X or X/Y), the genotypes *tra dsx^m/tra dsx^m* or *tra dsx^{mf}/tra dsx^{mf}* were intersexual showing the typical *dsx*-phenotype. All flies with genotypes *tra dsx^f/tra dsx^f* or *tra dsx^D/tra* had a male phenotype. In terms of gene interaction, *dsx^m* and *dsx^{mf}* are epistatic over *tra*, but *tra* is epistatic over *dsx^f* and *dsx^D*. This is easily understood when we recall that activity of *tra* needs a functional *dsx^f* to make a female, and an inactive *tra* needs a functional *dsx^m* to make a male.

(b) *Combinations involving dsx^D/ +*. The genotypes to be described in this section only affect X/X individuals; X/Y-flies are normal and fertile males. Each of the recessive mutations (*ix, tra-2, tra*), when heterozygous over its respective wildtype allele, interacts with *dsx^D* to produce a phenotype that is considerably more masculinized than *X/X; dsx^D/ +* (Figs. 1, 2). Based on this interaction, *dsx^D* was originally classified as an allele of *tra* (see *tra^D* in Lindsley & Grell, 1968), an error that was later corrected by Duncan & Kaufman (1975).

Homozygosity for *ix*, when combined with *dsx^D/ +*, produces a phenotype that is almost male (Figs. 1, 2). The inventory of male genitalia is complete and the structures are of almost normal size, the female genitalia are absent, the anal plates are male. Some residual female tendencies, however, are seen in traces of tergite 8 and in an intersexual position and shape of the sex comb bristles on the foreleg.

The same 'male' phenotype results when *dsx^D/ +* flies are also heterozygous for *tra-2* and *tra* (Figs. 1, 2). The general observation is that a reduction of wildtype alleles of *ix, tra-2* and *tra* enhances the masculinizing effect of *dsx^D/ +*. This result will be used to speculate about the interaction of *ix, tra-2* and *tra* with *dsx* (see Discussion).

4. Discussion

4.1. *The dsx locus*

The observations presented in this paper allow us to draw a picture of the structure and function of *dsx*. The locus apparently harbours two genetic functions, *dsx^m* and *dsx^f*, that can mutate independently. The different types of mutations are represented in Table 3. Recessive mutations in *dsx^m* and *dsx^f* fully complement each other in *trans* position. It is striking, however, that most of the newly induced mutations were of the *dsx^{mf}*-type abolishing both functions. We found 6 *dsx^{mf}*, 1 *dsx^f*, and 1 slightly dominant *dsx^{Dw}*-allele that is also female-specific. The same observation was made by Garen & Lepesant (pers. comm.) who isolated 17 *dsx^{mf}*, 3 *dsx^m* and 1 *dsx^f* allele. This probably means that *dsx^m* and *dsx^f* share a large region or many dispersed sites that are essential for both functions,

but also comprise separate domains that are only required for female development (*dsx^f*) or only for male development (*dsx^m*). It will be informative to see how the different mutations map relative to each other. In a pilot experiment, we were able to recover two wild-type (*dsx$^+$*) recombinants among 13626 offspring of females that were heterozygous for *dsx^m* and the *dsx^{mf}* allele of Hildreth (1965). This result and the arrangement of the flanking markers place *dsx^m* 0·03 cM to the left of *dsx^{mf}* (Grüter, 1983).

The function of the locus can be deduced from the mutant phenotypes, as summarized in Table 3. Recessive mutations that behave like null alleles show that *dsx^m* is required for male development, and *dsx^f* for female development. In the absence of either product, flies develop as intersexes with a phenotype that suggests that both sets of sex differentiation genes are simultaneously expressed in a cell. Shape and position of the bristles of the sex comb, e.g. are intermediate between the male and female type, similar to that shown in Fig. 3*d*. This suggests that the sex differentiation genes are constitutively active, and that the wild-type function of *dsx^m* is to repress the female set, that of *dsx^f* to repress the male set so that, in a normal fly, only one set of sex differentiation genes is expressed. The same conclusion was reached by Baker and his collaborators (review see Baker & Belote, 1983; Belote *et al.* 1985).

The dominant *dsx* mutations are particularly informative. So far, three spontaneous dominant alleles (*dsx^D, dsx^{Mas}, dsx^T*) and one weaker allele (*dsx^{Dw}*, induced by EMS) have been found. In combination with a wildtype *dsx$^+$* allele, the dominant mutations transform XX-zygotes into intersexes of the same type as do the recessive alleles; over *Df(dsx)*, *dsx^{mf}* or *dsx^f*, the strong spontaneous alleles transform XX-zygotes into pseudomales, but have no effect on XY-animals. They thus behave as constitutive mutations for *dsx^m* while at the same time lacking *dsx^f* function. The complementary dominant mutation, i.e. one that has a dominant *feminizing* effect on XY zygotes, has not been found. This fact has implications for our interpretation of the structure and regulation of the *dsx*-locus (see later).

Since *X/X; dsx^D/Df(dsx)* is a pseudomale, the intersexual phenotype of *X/X; dsx^D/ +* must be ascribed to the wild-type allele. In *X/X* animals, this wildtype allele is apparently expressed in the female mode, yielding the female-specific product of *dsx^f*. It is intriguing that *X/X; dsx^D/ +*, in which *dsx^m* and *dsx^f* are simultaneously expressed, produces the same intersexual phenotype as *X/X; dsx/dsx* in which no functional products are formed. If we introduce M and F to designate the functional products of *dsx^m* and *dsx^f*, respectively, we can say that M and F neutralize each other which leads to abolishment of both functions so that neither the male nor the female differentiation genes are repressed.

We remember that *dsx^D* in trans with all *dsx^{mf}*

Table 3. *The dsx-locus.*

Sex chromosomes	Types of mutations[a]				Deduced function of *dsx* in wildtype[b]			
	Designation of alleles and sexual phenotype				Pattern of activity at *dsx*-locus		Consequences on sex-specific differentiation	
	dsx (*dsx^{mf}*)	*dsx^m*	*dsx^f*	*dsx^D*	*dsx^{m+}*	*dsx^{f+}*	genes	sex
XX	⚥	♀	⚥	♂	—	F	Male set repressed	Female
XY	⚥	⚥	♂	♂	M	—	Female set repressed	Male

[a] Genetic analyses reveal two functions (complementation units) in *dsx*; the male-directing *dsx^m* and the female-directing *dsx^f* function (see text).

[b] In normal wild-type flies, *dsx* is regulated in such a way that either *dsx^m* or *dsx^f* is expressed. The respective products, M or F, are used the repress either the female set or the male set of sex differentiation genes. Absence of the required product results in intersexuality (⚥) due to expression of male and female differentiation genes. The allele *dsx^D* expresses M constitutively.

♀, female; ♂, male; ⚥, intersexual.

alleles and with *dsx^f* leads to male development; of the mutant alleles, only the male-specific *dsx^m* results in intersexual development of *X/X*; *dsx^D/dsx^m*. This indicates that an intact functional product of *dsx^f* is required to neutralize *dsx^D*. This neutralization could occur at the protein level. We arrive at this conclusion because the functional product of *dsx* appears to be a dimer or multimer, as suggested by the observation that *dsx^{584}* and *dsx^{501}*, 2 alleles of the *dsx^{mf}* type, complement in XY animals to give normal fertile males. It is therefore conceivable that the normal products of *dsx^m* and *dsx^f* which are both made in *X/X*; *dsx^D/+* can also aggregate, but such a heteromer would be non-functional.

Considering that $\widehat{XX}/Y . dsx^+$; *dsx^D/+* produces a phenotype that is essentially female, and $\widehat{XX}/Y . dsx^+$; *dsx^D/dsx^{Mas}* a phenotype that is essentially male, it appears as if F was titrated against M. If M and F were to form random aggregates, we would expect to find a minority of pure M-homomers in the former genotype, and a few pure F-homomers in the latter. These homomers could lead to some intersexuality. We have in fact observed that $\widehat{XX}/Y . dsx^+$; *dsx^D/+* are sterile 'females', and $\widehat{XX}/Y . dsx^+$; *dsx^D/dsx^{Mas}* 'males' display some weak female characteristics (see Results).

We are aware that our titration model is rather naive, but we were struck by its simplicity and accuracy in accounting for the observed phenotypes. It can also accommodate the phenotype of triploid intersexes carrying a *dsx^D*-allele (X/X;3A). These flies display a mosaic of male and female structures, as do regular triploid intersexes, and show no signs of the presence of a dominant masculinizing mutation, such as more and larger male structures of a *dsx*-phenotype. The mosaic character of regular triploid intersexes is thought to result from the ambiguous value of the X:A signal that is read by some cells as male, by

others as female (Baker & Belote, 1983; Steinmann-Zwicky & Nöthiger, 1985*a*, *b*). In *X/X*;*3A* with one dose of *dsx^D*, the constitutive expression of *dsx^m* in those cells that embark on the male pathway is, of course, without consequences; in the cells that respond by implementing the female program, the one *dsx^D* is counteracted by the two *dsx^+* alleles now expressing F, a situation that will lead to a female phenotype.

4.2. Regulation of dsx

How is *dsx* regulated so that in a normal male the locus expresses the male-determining M-function of *dsx^{m+}*, and in a normal female the F-function of *dsx^{f+}*? – The epistatic relations displayed by double mutants have been used to infer a genetic hierarchy with the gene *Sxl* at the top and *dsx* at the end of the cascade; the role of *tra-2*, *tra* and *ix* is to mediate between *Sxl* and *dsx* (for reviews see Nöthiger & Steinmann-Zwicky, 1985; Belote *et al.* 1985).

We want to discuss two questions: (1) do all these genes act in a cascade by forming a single chain? and 2) is *dsx* regulated at the transcriptional or post-transcriptional level?

If the genes were arranged in a single chain, e.g. *Sxl tra-2 tra ix* and if the purpose of the cascade was to achieve expression of either *dsx^m* or *dsx^f*, then recessive (lack of function) mutations in either *Sxl*, *tra-2*, *tra* or *ix* should result in XX animals becoming transformed into pseudomales, whereas dominant (constitutive) mutations in any one of these four genes should transform XY animals into pseudofemales. The predictions are fulfilled for recessive mutations in *Sxl*, *tra-2*, and *tra*, but not for the two alleles at the *ix*-locus; and dominant mutations with the expected phenotype are only known for *Sxl*, but not for *tra*, *tra-2* or *ix*. Therefore, we think that *tra-2*, *tra* and *ix* do

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not form a cascade, but are separately regulated and function in parallel; their concerted action achieves that *dsx* makes a functional F-product.

The genetic data which show two functions, *dsx*^{m} and *dsx*^{f}, are compatible with the existence of two cistrons. Formally, we can imagine that expression of *dsx*^{m} is the basic state, and that M represses F and other female-specific genes. When *dsx*^{m} is repressed (by *tra*$^+$ and other upstream genes), *dsx*^{f} can become active leading to F. Such a model, however, in which *dsx*^{f} is OFF as a consequence of *dsx*^{m} being ON, and is ON whenever *dsx*^{m} is OFF, is unlikely on several arguments:

- the constitutive mutations *dsx*^{D}, *dsx*^{Mas} and *dsx*^{T} only act in *cis* and do not prevent the correct expression of *dsx*^{f} by a wild-type allele in *trans*, as revealed by the intersexual phenotype of X/X; *dsx*^{D}/ + ;

- mutations that abolish the *m*-function (3 alleles, Garen & Lepesant, pers. comm.) do not lead to constitutive expression of *dsx*^{f} although the *f*-function is intact. In fact, no dominant constitutive mutations exist for *dsx*^{f} whereas four are known for *dsx*^{m};

- most mutations induced by EMS – which thus are mainly point mutations – abolish *m* and *f* simultaneously; the *m and f* functions appear small relative to the common function.

We want to propose that the *dsx*-locus is not regulated at the transcriptional level, but produces the same primary transcript(s) in XX and XY animals. This transcript has male-determining function. The female-determining transcripts could be made by splicing whereby *tra-2*$^+$ and *tra*$^+$ cooperate to remove or abolish the M-function. The ensuing product is non-functional, and acquires its functionality as F only after the product of *ix*$^+$ has further modified the transcript(s) or has combined with the *dsx* protein.

The following arguments lend support to this hypothesis:

- in the absence of positive regulatory signals, i.e. when none of the genes *Sx1*, *tra-2*, *tra*, *ix* are active, *dsx* expresses the male function. Thus, the M-function represents the basic state of *dsx*-expression;

- when *tra* **or** *tra-2* is inactivated by mutations, XX animals develop as pseudomales. Thus, both genes, *tra-2*$^+$ **and** *tra*$^+$ cooperate in XX animals to abolish the M-function, say by removing the *m*-domain. But the resulting product is non-functional; it is only 'demasculinized' and does not yet function as F, as revealed by mutations in *ix*. When this gene is mutated, but *tra-2*$^+$ and *tra*$^+$ are active, XX animals turn into intersexes. Thus, *ix*$^+$ is required in XX animals to convey F-function to the *dsx*-product after *tra-2*$^+$ and *tra*$^+$ have modified the basic M-product. (We know that *tra-2*$^+$ and *tra*$^+$ are active in X/X; *ix*/*ix* because such flies are intersexes whereas X/X; *ix*/*ix*; *tra*/*tra* or X/X; *ix tra-2*/*ix tra-2* are

pseudomales.) This view predicts that the genotype X/X; *ix*/*ix*; *dsx*^{D}/*dsx*$^+$, in which lack of *ix*$^+$ leaves the product of *dsx*$^+$ non-functional, should produce a pseudomale comparable to X/X; *dsx*^{D}/*dsx*^{mf}. This is practically the case although some residual activity of F is noticeable, probably due to *ix* being a leaky allele (see Figs. 1, 2);

- genotype X/X; *dsx*^{D}/*Df*(*dsx*) is a pseudomale showing that the mutant product of *dsx*^{D} is resistant to the action of *tra-2*$^+$, *tra*$^+$ and *ix*$^+$, three genes that, we conclude, must be active in X/X; *dsx*^{D}/ + since a functional F-product is formed by the wild-type allele. Reducing the dose of wild-type alleles at *tra-2*, *tra*, or *ix* reduces the amount of F-product in *dsx*^{D}/ + and shifts the sexual phenotype in the male direction (Fig. 2). Inasmuch as their effects are dose-sensitive, these genes behave like structural genes performing an enzymatic reaction.

We think that the presented evidence, although indirect, is more supportive of a post-transcriptional than of a transcriptional mode of regulation. The preliminary molecular data are compatible with both views (Belote *et al.* 1985).

The locus of *dsx* has recently been cloned (Belote *et al.* 1985). It is rather large comprising some 30 kb. If we extrapolate from the recombinational and molecular data of the *rosy* locus which has a genetic length of 0·005 cM and a molecular size of 4.1 kb (Coté *et al.* 1986), our recombination distance of 0·03 cM between *dsx*^{m} and *dsx*^{mf} corresponds to some 24 kb, an estimate that is in good agreement with the molecular data. The *tra*-gene, on the other hand, is at least 10 times smaller, with the relevant information being contained within some 2 kb (Butler *et al.* 1986; McKeown *et al.* 1987). Northern analysis of the *dsx* RNA show that sex-specific transcripts do in fact exist as anticipated on the basis of the genetic data. But the pattern of transcripts is more complex than expected, and sex-specific differences were so far only found in pupae and adults, but not in larvae (Belote *et al.* 1985). This latter result, however, must be due to insufficient sensitivity of the assays, since clonal analyses had shown that proper expression of *dsx* is required during larval development (Baker & Ridge, 1980). Direct observations also indicate that *dsx* is differentially expressed in male and female larvae. We conclude this because X/X; *dsx*^{D}/*Df*(*dsx*) larvae are phenotypically male with testes anlagen and male genital discs (unpubl. obs.). These pseudomales are indistinguishable from X/Y; *dsx*$^+$/*dsx*$^+$ normal male larvae, but differ from X/X; *dsx*$^+$/*dsx*$^+$ female larvae.

The cloning of the sex-determining genes is currently under way (Belote *et al.* 1985; Maine *et al.* 1985*a*, *b*; Butler *et al.* 1986; McKeown *et al.* 1987). The molecular probes will allow us to test the hypotheses put forward by developmental geneticists, and they will eventually reveal the regulatory network of the genes governing sex determination.

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