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Musca domestica, a window on the evolution of sex-determining mechanisms in insects

ANDREAS DÜBENDORFER*, MONIKA HEDIGER, GÉZA BURGHARDT and DANIEL BOPP

Institute of Zoology, University of Zürich, Switzerland

ABSTRACT The genetic cascades regulating sex determination of the housefly, *Musca domestica*, and the fruitfly, *Drosophila melanogaster*, appear strikingly different. The bifunctional switch gene *doublesex*, however, is present at the bottom of the regulatory cascades of both species, and so is *transformer-2*, one of the genetic elements required for the sex-specific regulation of *doublesex*. The upstream regulators are different: *Drosophila* utilizes *Sex-lethal* to coordinate the control of sex determination and dosage compensation, i.e., the process that equilibrates the difference of two X chromosomes in females versus one X chromosome in males. In the housefly, *Sex-lethal* is not involved in sex determination, and dosage compensation, if existent at all, is not coupled with sexual differentiation. This allows for more adaptive plasticity in the housefly system. Accordingly, natural housefly populations can vary greatly in their mechanism of sex determination, and new types can be generated in the laboratory.

KEY WORDS: *musca, drosophila, evolution, sex determination*

Introduction

Phenotypic differences between the sexes are the consequence of differential gene activities, and these are brought about by a primary sex-determining signal. Whatever the nature of this signal, it has to be transmitted to the many so-called sex realizer genes whose products are directly responsible for the expression of the sex-specific phenotype (Fig. 1).

In *Drosophila melanogaster,* the bottom-most switch gene mediating between the primary signal and the sex realizer genes is *doublesex (dsx)* (Burtis and Baker, 1989). The nascent transcript of *dsx* is differentially spliced to produce sex-specific mRNAs that code for either the female-specific DSX^F or the male-specific DSX^M proteins with their respective upregulating and downregulating effects on the yolk polypeptide genes (Coschigano and Wensink, 1993), and supposedly also on a host of other, yet unknown, sex-realizer genes. The perfect design of *dsx* as a terminal regulator that can set one of two alternative pathways in a developing organism immediately suggests that many species may make use of it for this purpose. Accordingly, Wilkins (1995) has proposed that in insects, *dsx* may be the most ancient and best conserved gene for sex determination, whereas the way it is regulated may have been subject to evolutionary changes. This may have led to the great variety of sex-determining mechanisms observed in recent insect species (Nöthiger and Steinmann-Zwicky, 1985).

In principle, one genetic or epigenetic signal which directly regulates sex-specific splicing of the *dsx* transcript would suffice for sexual differentiation, but the best understood insect system, *Drosophila melanogaster*, utilises a considerably more complicated signaling cascade between the primary signal and *dsx*, as shown in Fig. 2 (see also reviews by Schütt and Nöthiger, 2000; Bopp, 2001). To test the speculation that the original sex determination system was simpler, and that genetic variation of upstream elements led to the various mechanisms in recent insects, we set out to analyse sex determination in the housefly, *Musca domestica*. *Musca domestica* is a highly evolved insect species adapted to virtually all habitats of the earth. Evolutionists estimate that the genera Musca and Drosophila have been separate for some 120 million years. Here we show that Musca has preserved a mechanism of sex determination that in many respects is simpler than the one in Drosophila. Furthermore, and even more interestingly, single gene mutations in Musca change the type of sex determination in nature as well as in the laboratory (Fig. 2), revealing

Abbreviations used in this paper: Ag, Arrhenogenic; dsx, doublesex; F^D, $F^{Dominant}$; F^{man}, $F^{masculinizer}$; NOM, male with no *M* factor*; Sxl, Sex-lethal; tra, transformer; tra2, transformer-2.*

***Address correspondence to:** Dr. A. Dübendorfer. Institute of Zoology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich,Switzerland. Fax: +41-1635-6823. e-mail: andreas@zool.unizh.ch

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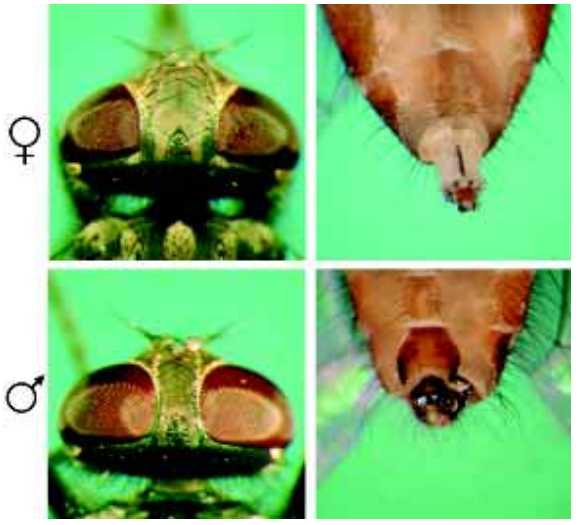

Fig. 1. Sexual dimorphism in the external morphology of *Musca domestica*. (Upper row) *Female head with wide eye distance (dorsal view), and female post-abdomen with ovipositor (ventral view).* **(Lower row)** *Male head with narrow eye distance (dorsal view), and postabdomen with male genital appendages (ventral view).*

possible ways evolution has gone to produce the great variety of sex-determining mechanisms observed among the insects.

The Primary Signal

Drosophila uses a titration mechanism of X-chromosomal against autosomal gene products to determine the sexual fate of the zygote. In the diploid zygote, presence of two X chromosomes constitutes a female signal, whereas presence of only one X directs male development (Cline and Meyer, 1996). Musca does it the simpler way: Males of the so-called standard strains have a Y chromosome that carries genetic factors *(M)* which set the male mode of differentiation (Fig. 3A). Females have no Y and thus no male sex-determining factors. The number of X chromosomes, two in females and one in males, is irrelevant in this process (Milani, 1967). From the observation of viable and fertile Y0 males one can conclude that the X is in fact dispensable as long as a Y chromosome is in the set. XX and XO animals, on the other hand, are fertile females. Thus, the minimal requirement for viability is the presence of one copy of either an X or a Y chromosome, which indicates that, apart from the male sex-determiners on the Y, the two heterosomes are genetically equivalent. Both, X and Y, are mostly heterochromatic, but differ in their morphological appearance (Hediger *et al.*, 1998b). More than 180 mutations have been analysed in Musca, and all of them were autosomal; not a single mutation maps to the X (Hiroyoshi, 1977; Milani, 1967; Wagoner, 1969). The equivalence of X and Y with respect to viability suggests no need for dosage compensation in the housefly.

Interestingly, wild housefly strains without Y chromosome also occur; both sexes are cytologically XX. These strains show sex-linked inheritance of mutations known to be autosomal, which indicates that the male determiner *M* is located on an autosome (Hiroyoshi, 1964; Rubini and Palenzona, 1967; Wagoner, 1969) (Fig. 3B). Such non Y-linked *M* factors have been found on all five autosomes (I-V) and even on the X chromosome.

The Main Switch Gene *"F"*

The existence of a mediating gene downstream of *M* became apparent when wild strains were isolated in which both sexes were homozygous for an autosomal *M* (McDonald *et al.*, 1978; Rubini *et al.*, 1972). Females of such strains are heterozygous for a dominant mutation F^D ($F^{Dominant}$ on chromosome IV) that promotes female development also in the presence of *M* (Fig. 3C). From this we deduce that activity of *F* is necessary for female development. In normal standard strains, activity of *F* is prevented by the presence of *M*, whereas in the F^D strains, *F* is mutated to a constitutive state and remains active in spite of *M*. *M* may thus code for a repressor of *F* and may have the property to translocate to different genomic sites. Another interesting hypothesis is that different *M* factors arose from dominant negative mutations in genes normally required for the activation of *F*.

If *F* is the female-determiner in the housefly, loss of *F* should result in maleness. This is actually the case: Schmidt *et al.* (1997a) isolated a loss-of-function or strong hypomorphic mutation of *F*, F^{man}, which, when homozygous in a chromosomally female embryo, reverses the sex and leads to perfect male development. This laboratory strain demonstrates a new type of sex determination in Musca without *M*, but with a dominant autosomal female determiner, F^+ (Fig. 3D). The strain, however, is handicapped by a maternal effect: F^{man}/F^+ offspring of F^{man}/F^+ mothers do not always develop as females, but often as intersexes, and sometimes even as fertile males, with temperature-dependent frequency (Schmidt *et al.*, 1997a). The same genotype F^{man}/F^+ is always female if derived from F^+/F^+ mothers. This demonstrates the importance of maternal *F* activity for the sexual development of the offspring.

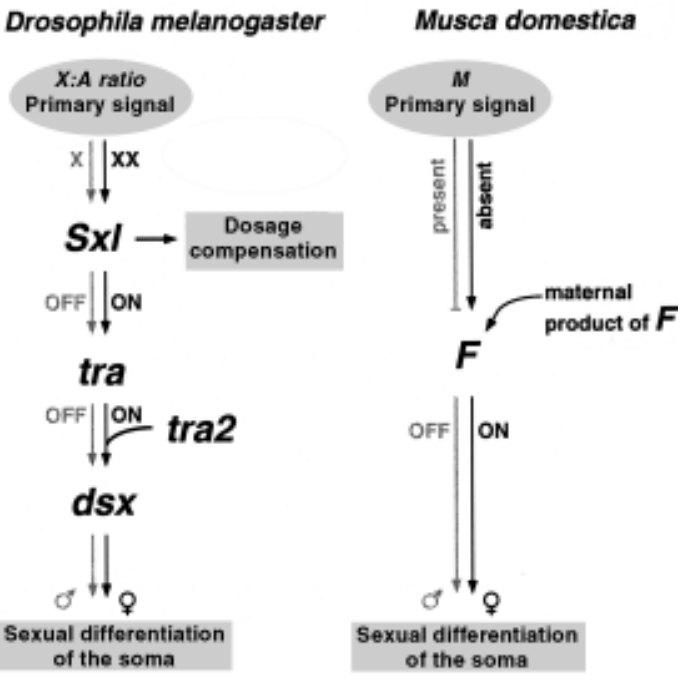

Fig. 2. Comparison of the sex-determining genetic cascades in *Drosophila melanogaster* and *Musca domestica*.

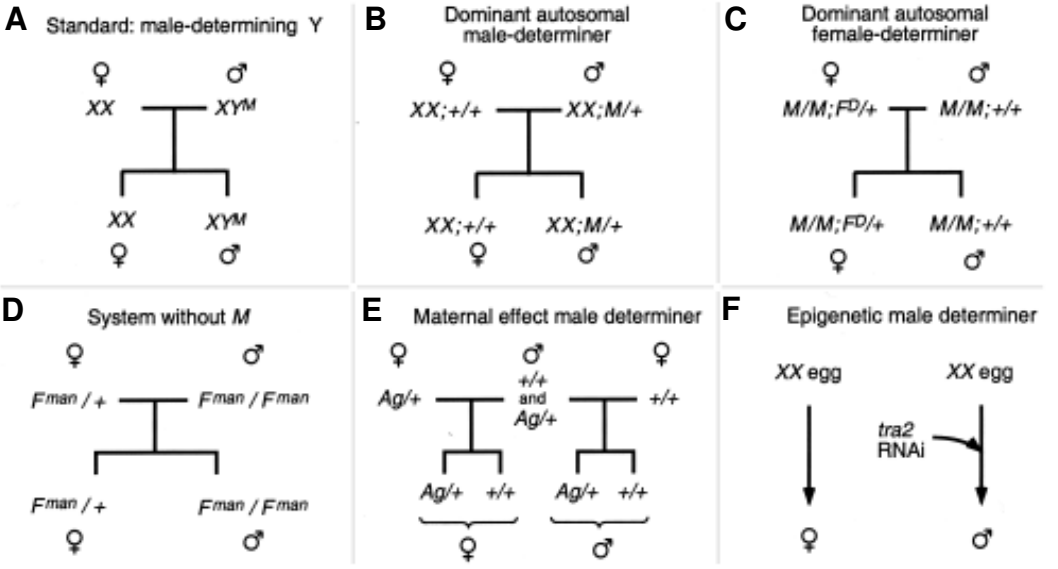

Fig. 3. Single gene mutations can change the sex-determining mechanism in the housefly. **(A)** *Standard XX-XY mechanism.* **(B)** *Sex determination by an autosomal dominant male-determiner.* **(C)** *Sex determination by an autosomal dominant female-determiner in the presence of* M. **(D)** *Sex determination by an autosomal dominant female-determiner in a system without* M. **(E)** *Sex determination by a male-determining maternal effect.* **(F)** *Epigenetic male sex determination by injection of* tra2 *dsRNA into genetically female embryos. A,B and C occur naturally; D,E and F were generated in the laboratory.*

The Maternal Contribution

In general, any zygote without *M* develops as a female, as long as the zygotic F^+ function is not disrupted by mutation. But there are exceptions to this rule: When pole cells (the germline progenitor cells) from a male embryo are transplanted to a genetically female embryo, these cells, although genetically male, integrate into the female germ line and produce normal eggs. Half of these carry the Y chromosome (or an autosomal *M,* depending on what donor strain was used), and the other half does not. If such eggs are fertilized by a male of the donor strain, 3/4 of the offspring are homo- or heterozygous carriers of *M,* and 1/4 are devoid of *M* and thus genetically female. Surprisingly however, also these aminals develop as fertile males (Dübendorfer, 2001; Hediger *et al.*, 1998a; Hilfiker-Kleiner *et al.*, 1994; Schmidt *et al.*, 1997b). This means that the presence of *M* in the maternal germ line imposes male development on all eggs that derive from this germ line, even in those that do not carry *M*. We refer to such males as "*NOM*-males" since they have no *M*. The case is different if the mother was *M*/+;F^D/+: their offspring that carry neither *M* nor F^D are now females albeit they, too, originate from a heterozygous *M* germline. Maternal F^D can thus rescue the offspring from the male-determining maternal effect, which suggests that maternal activity of *F* is a prerequitsite for female development. Yet, even this rule is not absolute. If both parents are experimentally modified to have chimeric germ lines, the mother carrying an *M*/+ germ line and the father an F^D/+ germline, offspring that carry neither *M* nor F^D are *NOM*-males, but offspring with the F^D allele are fertile females (Dübendorfer and Hediger, 1998). Taken together, these experiments demonstrate that female development requires *F* activity in the zygote, and that in non-experimental cases this activity requires maternal activity of the same gene *F*. Male development results whenever *F* cannot become active in the zygote, be it that *M* is present in the zygotic genome, or that maternal *F* was not functional due to either the presence of *M* or to mutational loss of function of *F* (F^{man}) in the germ line.

Most known *M* factors block *F* activity completely, but a special case has become known where the negative control is incomplete. The mutation *Ag (Arrhenogenic)* on chromosome I is a homozygous lethal, but in heterozygous condition, it affects neither sexual development nor viability in either sex. Heterozygous females, however, produce mostly fertile *NOM* sons and intersexes, whereas their *+/+* sisters produce exclusively daughters (Vanossi Este, 1971; Vanossi Este and Rovati, 1982). The *Ag* stock propagates with neither a Y chromosome nor an autosomal *M*: All males are *NOM*, and the females are either male-producing *Ag/+* or female-producing +/+. Since *Ag* maps to the same position as the known *M* on chromosome I, it may in fact be a variant of M^I, too weak for a zygotic male-determining effect, but strong enough to interfere with maternal *F* activity. By this mutation, Musca has passed from the zygotic into a maternal effect type of sex determination (Fig. 3E), a system that is standard for some other dipterans, e.g. the blowfly *Chrysomya rufifacies* (Ullerich, 1984).

The Bifunctional Switch *dsx* and its Regulation in *Musca*

If Wilkins' hypothesis (Wilkins, 1995) that regulatory systems evolve bottom-up is correct, the bottom-most gene of the sex-determining cascade, the genetic double switch *dsx*, should be the most conserved part of the system. This has proven true. On the basis of sequence homology with *D. melanogaster dsx*, we could isolate the *dsx* gene of *Musca domestica* and demonstrate sex-specific splicing as in Drosophila. *M/M;*F^D */+* flies have the female-specific splice variant of *dsx* RNA, and F^{man}/F^{man} *NOM* males the male-specific splice variant, respectively, which demonstrates that *dsx* is not under the direct control of *M*, but downstream of *F* (Hediger *et al.*, 2001).

F, as an upstream regulator of *dsx*, could be the equivalent to *tra (transformer)* in Drosophila (Fig. 4). The sex-specific functions of *F*, strictly female-determining when active, and male-determining when inactive, support this notion. Furthermore, the presence of TRA/TRA2 binding sites in the pre-mRNA of *dsx* suggest that its regulation is conserved, requiring TRA and TRA2 proteins as splicing factors to allow for female-specific *dsx* splicing. If this were true, and the mechanism analogous to the one in Drosophila, one would expect F protein to act jointly with non sex-specifically

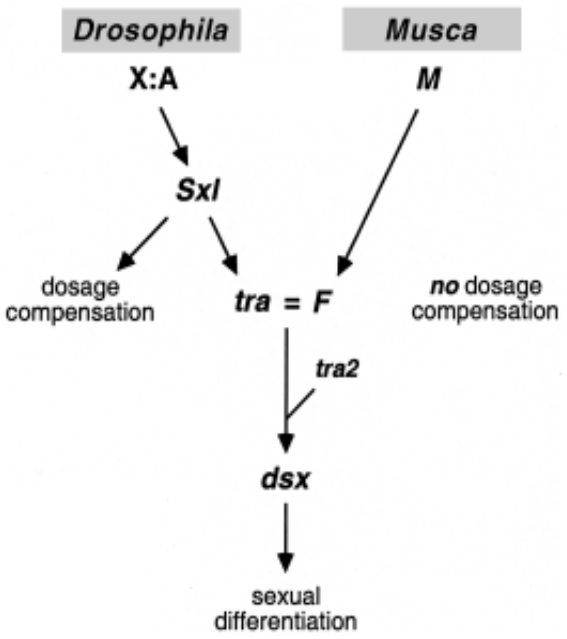

Fig. 4. Model for the divergent evolution of the sex-determining pathways of *Drosophila* and *Musca*. *While the bottom-most elements are shared, different upstream elements were introduced in* Drosophila *to coordinate the control of sex determination with dosage compensation.*

expressed TRA2 to direct female development. In contrast to *tra*, *tra2* is highly conserved in different phyla (Chandler *et al*., 1997; Dauwalder *et al*., 1996), which allowed us to isolate the *tra2* gene of Musca on the basis of sequence homology. As expected, the Musca homologue is equally transcribed in both sexes, but required for sex determination only in the female. When silenced by injection of *tra2* dsRNA, more than 90% of the female embryos become sex-reversed and develop as intersexes and even as fertile males expressing the male variant of *dsx* (Burghardt *et al*., 2001). Genotypic males are not affected, as predicted by the model. This experimental system demonstrates the possibility of an epigenetic control of *dsx* activity and thus a sixth mechanism of sex determination in the housefly (Fig. 3F).

Congruent Simplicity and Evolving Complexity

As far as our studies tell us, the basics of sex determination, namely the bifunctional switch *dsx* and its regulation, are highly conserved in Drosophila and Musca. We propose that *F* in Musca represents the direct upstream regulator of *dsx* and thus functionally corresponds to *tra* in Drosophila (Fig. 4). Upstream of this level, however, the two pathways are clearly divergent. The cascade in *Drosophila melanogaster* is extended by one more level of control, *Sex-lethal (Sxl)*. Once the X:A ratio is assessed, this gene is engaged in the coordinate control of all aspects of sexual development including X-linked gene expression (Fig. 4). Inappropriate function of this gene results in lethality because the X chromosome dosage is not compensated correctly (for reviews see Cline and Meyer, 1996; Meller, 2000).

Musca domestica is different: Though it has a well-conserved *Sxl* gene in the genome, this gene is expressed in both sexes and is apparently not involved in sex determination (Meise *et al*., 1998). Moreover, the function of *Sxl* to control dosage compensation in Drosophila may not be required in Musca, since X and Y chromosomes harbor the same essential genes. Rather, it seems that the role for *Sxl* as a coordinator of the control of sexual development and X-chromosomal gene expression is unique to the genus Drosophila and represents a more recent acquisition in the evolution of sex-determining mechanisms (Bopp *et al*., 1996; Penalva *et al*., 1996; Erickson and Cline, 1998). The function of *Sxl* in Musca and other dipteran species remains to be investigated (Müller-Holtkamp, 1995; Saccone *et al*., 1998; Sievert *et al*., 1997).

With our present knowledge, the way Musca determines sex seems considerably simpler than the way it is done in Drosophila, but our view may be biased by the far lower degree of genetic and molecular analysis in the housefly. Still, it is conceivable that an ancestral type of sex-determining mechanism, involving *tra*, *tra2* and *dsx*, was similar to what we still see in the housefly. Both genera have specialized in the past 120 million years. Drosophila did so by using the quantitative signal of the relative number of X chromosomes and by recruiting *Sxl* as a mediator to coordinate the control of the novel requirement for X chromosome dosage compensation with the control of sexual development via *tra*. Musca, on the other hand, engaged a dominant male-determiner to control *F*, the functional equivalent of *tra*. One may wonder why Musca did not make use of the simpler possibility of regulating *dsx* with a functional and a non functional allele of *F*, as illustrated by Fig. 3D. The answer lies in the dosage effect of maternal *F* activity. With only one dose of F^+, a zygote can, in principle, develop as a normal female if it derives from a mother with two functional *F* alleles. In contrast, only one dose of F^+ in the maternal germ line does not suffice to furnish the egg with enough *F* product to activate zygotic *F* reliably. This demonstrates the existence of an autoregulatory function of *F*, which appears highly dose sensitive. In the light of these circumstances, a system with two F^+ alleles and an independent primary signal that either allows activity of both *F* alleles, or prevents activity of both, is a more reliable switch for sex determination.

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