The Mutation *masculinizer* (*man*) Defines a Sex-Determining Gene With Maternal and Zygotic Functions in *Musca domestica* L.

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ABSTRACT

In *Musca domestica*, the primary signal for sex determination is the dominant factor *M*, which is assumed to regulate a postulated female-determining gene *F*. Presence of *M* prevents expression of *F* so that male development ensues. In the absence of *M*, *F* can become active, which dictates the female pathway. The existence of *F* is inferred from *F^D*, a dominant factor that is epistatic to *M*. We describe a new mutation *masculinizer*, which has all the properties expected for a null or strongly hypomorphic allele of *F*: (1) it maps to the same chromosomal location as *F^D*, (2) homozygous *man/man* animals develop as males, (3) homozygous *man/man* clones generated in *man/+* female larvae differentiate male structures, (4) *man* has a sex-determining maternal effect. About a third of the morphological males synthesize yolk proteins, which indicates that they are intersexual in internal structures. The maternal effect of *man* is complete in offspring that derive from homozygous *man/man* pole cells transplanted into female hosts. In this case, all *man/+* progeny become fertile males that do not produce yolk proteins. A sex-determining maternal effect has previously been demonstrated for *F^D*. Like *F*, maternal *man⁺* is needed for zygotic *man⁺* to become active, providing further evidence that *man* is a loss-of-function allele of *F*.

MOST animal species are sexually dimorphic and produce males and females. To achieve this sexual differentiation, organisms use a variety of mechanisms. In standard strains of the common housefly *Musca domestica*, sex is determined by the presence or absence of the Y chromosome, which carries a male-determining factor, *M* (PERJE 1948). In other strains, females and males have two X chromosomes, and *M* is located on one of the five autosomes or on the X chromosome (INOUE *et al.* 1983, 1986; INOUE and HIROYOSHI 1984). In these strains, the male represents the heterogametic sex (*M/+*). The *M* factors, whether located on the Y chromosome or on another chromosome, seem to be functionally equivalent, which suggests that *M* is a mobile element (GREEN 1980; NÖTHIGER and STEINMANN-ZWICKY 1985; TRAUT 1994). The autosomal sex determination mechanism is further modified in strains where both sexes are homozygous for *M*, but females are heterozygous for a dominant factor *F^{Dominant}* (*F^D*) that is epistatic to *M* and induces female development even in a background of one or several *M* factors (RUBINI 1967; McDONALD *et al.* 1978; DÜBENDORFER *et al.* 1992).

Sex can also be determined maternally without the presence of zygotic *M* or *F^D*. In these strains, mutant mothers force their genotypically female offspring to develop as sexual mosaics or males. So far, two such mutations are known. The dominant mutation *Arrhen-*

genic (*Ag*) is located on chromosome I (VANOSSI ESTE and ROVATI 1982) and has a variable penetrance. Since homozygous *Ag* flies have never been observed, *Ag* may have a recessive lethal effect or is closely linked to a recessive lethal mutation. Heterozygous *Ag/+* females are arrhenogenic, *i.e.*, produce mostly intersexes and males, whereas *+/+* mothers are thelygenic, *i.e.*, produce only daughters. The recessive mutation *transformer* (*tra*) is localized on chromosome IV (INOUE and HIROYOSHI 1986). Homozygous *tra/tra* females are arrhenogenic, while heterozygous *tra/+* females produce mostly female offspring. In both cases, *Ag* and *tra*, the intersexes are mosaics consisting of female and male parts side by side (VANOSSI ESTE and ROVATI 1982; INOUE and HIROYOSHI 1986), comparable to the triploid intersexes (2X:3A) of *Drosophila melanogaster* (BRIDGES 1921). The male offspring of either heterozygous *Ag/+* or homozygous *tra/tra* females are devoid of *M*. Due to their female genotype, we call these males NOM males (for "no *M*"; HILFIKER-KLEINER *et al.* 1994).

These various mechanisms of sex determination in *M. domestica* could reflect variations of a basic system (NÖTHIGER and STEINMANN-ZWICKY 1985) where a key gene is regulated by a primary signal with the help of maternal components. In *M. domestica*, *M* appears as the primary signal that is constitutively expressed and functions to prevent the activity of a postulated key gene *F* (NÖTHIGER and STEINMANN-ZWICKY 1985; INOUE and HIROYOSHI 1986; HILFIKER-KLEINER *et al.* 1993). The existence of *F* is inferred from the dominant factor *F^D*, which is interpreted as a constitutive allele of *F* that

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escapes inactivation by *M*. In the female germ line of genetic mosaics, *M* has a masculinizing maternal effect that can be overcome by *F^D* (HILFIKER-KLEINER *et al.* 1994). This suggests that *F* has to be expressed in the female germ line and that its products are necessary for the activation of the zygotic *F⁺* allele.

In this study, we characterize the recessive mutation *masculinizer* (*man*), a component involved in sex determination of *M. domestica*. We mapped *man* to chromosome IV, in the same region as *F^D*. Heterozygous *man/+* animals are females, whereas homozygous *man/man* zygotes develop as males. We further show that female differentiation requires *man⁺* function throughout development, and that homozygosity for *man* induced at any time in individual cells reverts the sex of such cells to maleness. Additionally, *man* has a maternal effect that transforms genotypically female offspring (*man/+* or *+/+*) into intersexes and males. We present evidence suggesting that *man* is a strongly hypomorphic allele of *F*.

MATERIALS AND METHODS

Rearing of flies: Stocks and flies for mass crosses were kept in acrylic glass cages (14 × 14 × 19 cm) at 23° and fed on water, sucrose and milk-powder. Since the flies prefer to lay their eggs in dark places, black cylindrical plastic boxes (ordinary film boxes), half way filled with larval rearing medium and topped with a punched lid, were used to collect the eggs. Larvae were reared at 25° and 70% relative humidity at a 12-hr dark/light cycle on a diet prepared according to the following recipe: 900 ml water, 480 g wheat bran, 60 g wheat flour, 40 g milk-powder, 20 g brewer's yeast, and 20 ml ethanol containing 0.67 g Nipagin and 1.33 g Nipazol as fungicides.

For single pair crosses, flies were kept in transparent plastic beakers (180 ml) and fed on water, sucrose and milk-powder until females were ready for egg laying. The milk-powder was then replaced by larval rearing medium on which the eggs were deposited.

Genotypes: Genes, mutations, symbols and linkage maps are described by MILANI (1967) and HIROYOSHI (1977). The autosomal markers used in this study are *ac* (*ali curve*) on linkage group I; *ar* (*aristapedia*) on linkage group II; *bwb* (*brown body*), *ge* (*green eyes*) and *pw* (*pointed wings*) on linkage group III; *Ba* (*Bald abdomen*), *cyw* (*curly wings*) and *ye* (*yellow eyes*) on linkage group IV; *ocra* (*ochre eyes*), *apt* (*apterous*) and *snp* (*snip wings*), kindly provided by J. G. SCOTT, Cornell University) on linkage group V. A superscript (*X*, *Y* or roman number) was used to specify the linkage group, e.g., *M^{III}* for the male determiner on linkage group III.

Four strains with standard XX-XY sex determination (strains 1–4), four strains with autosomal sex determination (strains 5–8) and one strain with maternal sex determination (strain 9) were used: (1) XX-XY; *+/+*, (2) XX-XY; *ac*; *ar*; *bwb*; *ye*; *snp*, (3) XX-XY; *Ba*/*+*, (4) XX-XY; *ocra apt*, (5) XX; *bwb ge pw⁺ M^{III}/bwb ge pw*, (6) XX; *bwb pw⁺ M^{III}/bwb pw*, (7) XX; *bwb⁺ M^{III}/bwb*, (8) XX; *bwb ge M^{III}/bwb ge M^{III}; F^D Ba*/*+*, (9) XX; *Ag*/*+*; *bwb*. Females of the genotype XX; *Ag*/*+*; *bwb* are arrhenogenic, i.e., produce intersexes and NOM males. Daughters are obtained from XX; *+/+*; *bwb* mothers, which are also present in the strain (VANOSSI ESTE and ROVATI 1982).

Origin of the *man* chromosome: The stock XX; *bwb ge pw⁺ M^{III}/bwb ge pw* produces males carrying the *bwb ge pw⁺ M^{III}* chromosome (normal wings), and females homozygous for

bwb ge pw (pointed wings). In this stock, males with the female wing phenotype (*pw*) spontaneously occurred. These males were sterile, and so we crossed their brothers and sisters *inter se*. In the next generation, we found again sterile *pw* males. After some generations, however, some *pw* males became fertile and, when crossed with *bwb ge⁺ pw* females (stock 6), produced offspring that were all female. This indicated that the *pw* males were not crossover products with a genotype *M^{III} pw/pw*, but carriers of a new, recessive mutation that we named *masculinizer* (*man*).

Linkage group analysis: The linkage group of *man* was determined by crossing males of the *man* stock to tester females that were either homozygous for *ac* and *ar* (strain 2), or homozygous for *bwb⁺* (strain 1), or heterozygous for *Ba* (strain 3), or homozygous for *apt* (strain 4). The heterozygous F₁ females were then backcrossed to tester males (XY, from strains 1–4). Of the F₂ females carrying the marker(s), 20 were individually tested for *man* by crossing them to *man* males of the stock. Presence of males in the F₃ was indicative of the F₂ females being heterozygous for *man*.

Clonal analysis: We crossed XX; *bwb* females (stock 7) to XX; *bwb⁺*; *man/man* males to produce heterozygous *bwb/+*; *man/+* animals. These larvae were reared at 25° and X-irradiated at 48, 72, 96 and 120 hr after oviposition, respectively. A Philips MG 160 X-ray machine at 150 kV, 14 mA, 2-mm Al filter, 1-mm acrylic glass, at an irradiation distance of 25.0 cm was used. The dose was 10 Gy for all larvae except for the 48-hr batch that, due to higher sensitivity (HILFIKER-KLEINER *et al.* 1993), was given only 8 Gy.

Controls consisted of unirradiated larvae of the same genotype, of unirradiated and irradiated *bwb/+* animals derived from XX; *bwb* females (stock 7) crossed to XY; *+/+* males (stock 1), and of unirradiated and irradiated wild-type larvae (stock 1).

The flies were examined 1 day after emergence. Abdominal carcasses, external genitalia and wings were spread on microscopic slides and mounted in Euparal. The frequency and the sexual phenotype of the clones in sexually dimorphic regions of the abdomen were assessed with a compound microscope. The frequency of *bwb* clones was determined on the wings only. Clone sizes were neglected because they are difficult to measure due to the scarcity of marker structures in the genitalia, and they are not important for our question.

Temperature sensitivity: Adult *Ba man⁺/Ba⁺ man* females were crossed to *man/man* males either at 22° or at 29°. Their eggs were either left to develop at the same temperature or shifted to the alternative temperature either at 0–2 hr or 96 hr after oviposition. The frequency of males and intersexes among the *man/+* offspring was determined.

Aging effect: The frequencies of females, intersexes and males with the genotype *man/man⁺* and *man⁺/man⁺* were determined among the offspring of heterozygous *Ba man⁺/Ba⁺ man* females that were either 6–9 or 10–13 days old. Seven groups of 10 females of the genotype *ar; ge pw; Ba man⁺/Ba⁺ man* were mass-crossed to males of a translocation stock. In this cross, the relevant class of offspring, i.e., those without *M*, are recognizable by their *ar* phenotype. The reason for this is that the males in the translocation stock are heterozygous for a reciprocal translocation, *T(Y; II)2,ar⁺/+*, involving the Y chromosome and the second chromosome (M. HEDIGER, unpublished results). The *M* factor and *ar⁺* are pseudolinked so that a wild-type phenotype (*ar⁺*) indicates presence of *M^I*, and a mutant phenotype (*ar*) absence of *M^I*.

Analysis of yolk proteins: Adult flies were fed for 5 days with sugar water and milk powder. To check for male fertility, a single male was put together with three standard females for 1 day. At the sixth day, ~1 µl of haemolymph was taken from the slightly CO₂-anesthetized male. The haemolymph

was then processed as described by BOPP *et al.* (1991). We used a polyclonal anti-yolk-protein-antibody of *M. domestica* (1:1000 dilution in TBS/0.05% Tween-20), kindly provided by Dr. T. ADAMS, Fargo, ND. For subsequent detection of antigen-antibody complexes on the blot, we used the alkaline phosphatase-conjugated anti-rabbit kit from Promega.

Transplantation of pole cells: Transplantations were done according to the protocol described by HILFIKER-KLEINER *et al.* (1994), except that porcine faeces were used instead of standard medium for rearing the larvae (SCHMIDT and BÄCHLI 1996). This made the use of helper larvae unnecessary. The larvae were grown at 23°.

Series 1: Genotype of host embryos: XX; +/+; +/+ or XY; +/+; +/+. Genotype of donor embryos: XX; *bwb*; *Ba man*⁺/*Ba*⁺ *man* or XX; *bwb*; *man/man*.

Series 2: Genotype of host embryos: XX; *ac*; *ar*; *bwb*; *ye*; *snp* or XY; *ac*; *ar*; *bwb*; *ye*; *snp*. Genotype of donor embryos: XX; *bwb*; *Ba man*⁺/*Ba*⁺ *man* or XX; *bwb*; *man/man*.

Female hosts of both series were crossed to NOM males from the Ag stock (genotype Ag/+; *bwb* or +/+; *bwb*). Male hosts were crossed to standard XX; *bwb* females. The donor-derived offspring were recognized by their body color.

Combinations of *man* with *M* and *F*^D: A series of crosses was performed to obtain *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge pw*; *Ba F*^D/*Ba*⁺ *man* females and *bwb ge pw*/*bwb*⁺ *ge pw*; *Ba F*^D/*Ba*⁺ *man* females. We crossed homozygous *bwb ge pw*/*bwb ge pw* females to heterozygous *bwb*⁺ *ge*⁺ *pw*⁺ *M*^{III}/*bwb ge pw*⁺ *ge*⁺ *pw*⁺ males. *M*^{III} is very tightly linked to *pw*⁺ (HILFIKER-KLEINER *et al.* 1994). In the F₁, the resulting heterozygous *bwb*⁺ *ge*⁺ *pw*⁺ *M*^{III}/*bwb ge pw* males were crossed to females of the genotype *bwb ge pw*⁺ *M*^{III}; *Ba F*^D/+. Of the resulting F₂, we selected the *bwb* females with the genotype *bwb ge pw*⁺ *M*^{III}/*bwb ge pw*; *Ba F*^D/+ and crossed them back to their fathers (*bwb*⁺ *ge*⁺ *pw*⁺ *M*^{III}/*bwb ge pw*). In the F₃, we obtained three different types of females: (1) *bwb ge pw*⁺ *M*^{III}/*bwb ge pw*; *Ba F*^D/+, (2) *bwb ge pw*; *Ba F*^D/+, (3) *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*⁺ *M*; *Ba F*^D/+. Type 3 females were discarded. Females of type 1, crossed to homozygous *bwb*⁺ *ge*⁺ *pw*⁺ *man/man* males, yielded the desired *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*⁺; *Ba F*^D/*Ba*⁺ *man* females. To produce the *bwb ge pw*/*bwb*⁺ *ge*⁺ *pw*; *Ba F*^D/*Ba*⁺ *man* females, we crossed the type 2 females to *bwb*⁺ *ge*⁺ *pw*; *man/man* males.

To construct *M*^{III}/+; *man/man* males, we crossed *bwb ge pw*⁺ *M*^{III}; *Ba F*^D/+ females to *bwb*⁺ *ge*⁺ *pw*; *man/man* males. The F₁ *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*; *man*/+ males were then crossed to *bwb*⁺ *ge*⁺ *pw*; *Ba man*⁺/*Ba*⁺ *man* females. In the F₂, we obtained three types of males: (1) *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*; *Ba man*⁺/*Ba*⁺ *man* males, (2) *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*; +/*man* males and (3) *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*; *man/man* males. Since types 2 and 3 are not distinguishable, we crossed single males to *bwb*⁺ *ge*⁺ *pw*; *Ba F*^D/*Ba*⁺ *man* females. Occurrence of *Ba*⁺ females in the offspring was proof that the father was of the genotype *bwb ge pw*⁺ *M*^{III}/*bwb*⁺ *ge*⁺ *pw*; +/*man*.

RESULTS

Isolation and localization of *man*: In one of our stocks, a spontaneous recessive mutation occurred that transforms genotypically female embryos into fertile males. These males, when crossed to wild-type females, produce only daughters, showing that they are devoid of the primary male-determining factor *M*. We named the mutation *masculinizer (man)* and established a stock with female heterogamety where females are heterozygous and males are homozygous for *man*.

Linkage analysis showed that *man* is located on chromosome IV. We further mapped *man* relative to *Ba*.

Among the offspring of heterozygous *Ba man*⁺/*Ba*⁺ *man* females, crossed to homozygous *man/man* males, 1482 *Ba* females, 1532 *Ba*⁺ males, 116 *Ba* intersexes and four *Ba* males were found. The intersexes were mosaics of female and male areas (Figure 1, B and C; see also below). We crossed the four *Ba* males individually to standard +/+ females. The resulting daughters were then backcrossed to males of the *man* stock. We found that the *Ba* daughters only produced females, whereas *Ba*⁺ daughters yielded male and female offspring. This shows that the four *Ba* males had been trans-heterozygous for *man* and *Ba*, and that their male phenotype was the consequence of a maternal effect (see below), but not of recombination. Thus, *man* is tightly linked to *Ba* (no recombinants out of 3134 offspring), a property shared with *F*^D (MCDONALD *et al.* 1978). Since homozygosity for *Ba* leads to lethality (M. HEDIGER, unpublished results), it became possible to use the *Ba* chromosome as a balancer. We established a stock in which the *Ba* phenotype is indicative of heterozygosity of *man* (*Ba man*⁺/*Ba*⁺ *man*), and *Ba*⁺ for homozygosity of *man* (*Ba*⁺ *man*/*Ba*⁺ *man*).

Dominant maternal effect of *man*: We could show that the *Ba* males, and probably also the intersexes that were sterile and could not be crossed, were heterozygous for *man*. Such sexual mosaics and males are only obtained when the mother was heterozygous for *man*, irrespective of the father's genotype. When crossed to *man/man* males, standard +/+ mothers produce only female offspring. This means that the occurrence of intersexes and males is not due to a slight dominant zygotic effect of *man*, but rather that one dose of maternal *man*⁺ product is not always sufficient to allow female development of the offspring. Thus, *man* shows a dominant maternal effect that can transform genotypically female offspring (XX; +/+ or XX; *man*/+) into intersexes and fertile males. The severity of this masculinizing maternal effect is dependent on the age of the mother and on temperature.

Aging effect: Young mothers of the genotype *man*/+ yield more daughters than intersexes and males (Table 1). As the mothers become older, the masculinizing maternal effect increases drastically, resulting in over 90% intersexual and male offspring.

Temperature-sensitivity: When kept at 22° throughout development, most of the heterozygous *man*/+ offspring that derived from a cross of heterozygous *man*/+ females and *man/man* males develop as females, and only few animals develop as intersexes and males (Table 2). At 29°, about five times more heterozygous *man*/+ animals develop as intersexes and males.

The temperature-sensitive period was defined by shifting the offspring during embryonic or larval development (Table 2). Of the animals shifted immediately after oviposition from 22° to 29°, a large proportion of the heterozygous *man*/+ individuals developed as females, mimicking the situation of continuous devel-

FIGURE 1.—Sexual mosaicism in the postabdomen of the housefly. (A) Male structures differentiated by a clone of *man/man* cells in a heterozygous (*man/+*) female. The clone was generated by mitotic recombination in the second larval instar. (B and C) Intersexual development (mosaic type) of *man/+* animals as a consequence of the maternal effect of *man*. C, Female anal cerci; dA, female dorsal anal plate; E, epandrium of the male genitalia; H, hypandrium of the male genitalia; S, surstylus of the male genitalia; St8, female abdominal sternite 8; T8, female abdominal tergite 8. Bars, 100 μ m.

opment at 22° (Table 2). Similarly, most of the animals shifted late in development from 22° to 29° developed as females. If the parents were kept on high temperature, a shift-down of their offspring at any developmental stage resulted in a still high frequency of heterozygous *man/+* intersexes and males. We conclude that the frequency of intersexes and males among the offspring of *man/+* females depends mainly on the temperature that the mother experienced before oviposi-

tion, which is consistent with the observed maternal effect.

Transplantation of pole cells: Since intersexes and males occur among the offspring of heterozygous *man/+* females, a further reduction in the dose of maternal *man*⁺ genes might increase the masculinizing maternal effect and result in exclusively male offspring. To test this, we transplanted pole cells of the genotype *XX; bw; man/man* or *XX; bw; Ba +/+ man* into embryos

TABLE 1

The maternal effect of *man* is dependent on the age of the *man/+* mother

Age of mother	<i>man/man</i> ⁺ offspring (<i>Ba</i> ⁺) ^a			<i>man</i> ⁺ / <i>man</i> ⁺ offspring (<i>Ba</i>) ^a		
	Females (%)	Intersexes and males (%)	<i>n</i>	Females (%)	Intersexes and males (%)	<i>n</i>
6–9 days	65.8	34.2	73	78.0	22.0	68
10–13 days	9.8	90.2	41	7.0	93.0	43

^a Offspring of heterozygous *XX; ar; Ba man*⁺/*Ba*⁺ *man* females crossed to *X/Y^pIP^p, ar⁺; IP^pY^d/ar; man*⁺/*man*⁺ males (see MATERIALS AND METHODS for details). Only offspring without *M* (*ar/ar*) were considered. Males and intersexes were pooled, since the number of those animals that were intersexual in internal structures could not be determined. *n*, number of animals examined.

TABLE 2
Temperature-sensitivity of *man*

Stage of shifted animals	Temperature before/after shift (°)	<i>man/man</i> ⁺ (<i>Ba</i>) ^a		<i>n</i>
		Females (%)	Intersexes and males (%)	
Embryos	22/29	93.7	6.3	159
	29/22	50.6	49.4	352
3rd instar	22/29	70.4	29.6	192
	29/22	55.4	44.6	130
Control	22	89.4	10.6	470
	29	42.3	57.7	813

^a Offspring of heterozygous *Ba man*⁺/*Ba*⁺ *man* females crossed to homozygous *man/man* males. Mothers at the period of high reproductive activity (5–12 days old) were kept at either 22° or 29°, and offspring were shifted either 0–2 hr after oviposition (embryos) or in 3rd instar. The homozygous *man/man* offspring were always males and are not listed in this table. *n*, number of animals examined.

of the genotype XX; +/+ or XY; +/+. Of 595 injected embryos, 140 (23.5%) survived to adulthood. Of the 68 females, 10 had integrated donor pole cells that gave rise to offspring. Of the 46 males that were crossed to wild-type females, five had a mosaic germ line.

The data, summarized in Table 3, demonstrate first that the homozygous *man/man* germ cells are able to form eggs (line D), and, second, that the resulting *man*/+ offspring consisted exclusively of males. When these males were crossed to standard +/+ females, they produced only daughters. As in normal heterozygous females, the offspring of heterozygous *man*/+ germ cells in a wild-type female host consisted of females, intersexes and males (line B). As expected, integration of heterozygous *man*/+ or homozygous *man/man* pole cells into wild-type male hosts resulted in exclusively female offspring, when crossed to standard +/+ females (lines A and C).

Our data show that *man*⁺ function is required in the female germ line not for oogenesis, but for female sexual development of the zygotes.

Zygotic effect of *man*: The masculinizing effect exerted by young *man*/+ mothers is somewhat stronger in heterozygous *man*/+ offspring than in +/+ offspring (Table 1). This points to a slight zygotic effect of *man* that suggests an interaction of maternal *man*⁺ product with the zygotic *man*⁺ allele. In older females, the maternal effect of *man* is much stronger, overruling the zygotic effect of *man* that results in equal frequencies of +/+ and *man*/+ intersexes and males among the offspring. A slight dominant effect of *man* is also indicated by the occurrence of 20.2% intersexes among the heterozygous *man*/+ offspring of heterozygous *M*/+; *F*^D/+ females crossed with *man/man* males (Table 4, series 7). When the same females were crossed to wild-type males, all +/+ offspring developed as females (Table 4, series 8).

Interactions between *man*, *M* and *F*^D: By transplantation of pole cells, HILFIKER-KLEINER *et al.* (1994) showed that *M* exerts a maternal effect: it predetermines all embryos with a female genotype to develop as males. They further showed that presence of *F*^D in the female germ line prevents the masculinizing maternal effect of *M* so that zygotes with a female genotype, devoid of *M* and *F*^D, now develop as females.

To test the interactions between *M*, *F*^D and *man* we constructed heterozygous *Ba F*^D/*Ba*⁺ *man* females and crossed them to *pw*⁺ *M/pw*; +/+ males. Almost all of the *Ba*⁺ *man*/+ offspring without *M* and *F*^D developed as females (Table 4, series 3), whereas intersexes and males were obtained from heterozygous *man*/+ mothers without *F*^D (Table 4, series 4 and 5). This clearly shows that the masculinizing maternal effect of *man* is weakened by the maternal effect of *F*^D. If heterozygous *F*^D/+ females or *man*⁺/*man*⁺ females were crossed with homozygous *man/man* males, only female offspring resulted (Table 4, series 1 and 2).

The frequency of intersexes could be increased by introducing an *M* factor into the female germ line: females of the genotype *M*^{III}/+; *F*^D/*man* (Table 4, series 6) produced more intersexual offspring when crossed

TABLE 3
Transplantation of *man/man* and *Ba man*⁺/*Ba*⁺ *man* pole cells into wild-type hosts

	Genetic sex of donor pole cells ^b	Sex and number of host (n)	Adult hosts were crossed to	No. of host-derived offspring ^a		No. of donor-derived offspring ^a					
				Females	Males	<i>Ba</i> females	<i>Ba</i> ⁺ females	<i>Ba</i> intersexes ^c	<i>Ba</i> ⁺ intersexes ^c	<i>Ba</i> males	<i>Ba</i> ⁺ males
A	XX; <i>Ba/man</i>	XY; +/+ (1)	XX; +/+	58	63	4	2	0	0	0	0
B	XX; <i>Ba/man</i>	XX; +/+ (6)	NOM males ^d	422	0	33	31	43	55	9	7
C	XX; <i>man/man</i>	XY; +/+ (4)	XX; +/+	121	145	0	148	0	0	0	0
D	XX; <i>man/man</i>	XX; +/+ (2)	NOM males ^d	229	0	0	0	0	0	0	38

^a Donor- and host-derived offspring were distinguished by their body color (see MATERIALS AND METHODS).

^b The genotype of the donor pole cells is inferred from presence or absence of *Ba*.

^c The intersexes are mosaics of female and male regions.

^d The NOM males were of genotype XX; *Ag*/+; *bwb* or XX; +/+; *bwb*.

TABLE 4
Interactions of *M*, *F^D* and *man*

Series	Maternal genotype ^a	Paternal genotype ^a	Number and sex of <i>man/man</i> ⁺ offspring			Frequency of intersexes and males (%)
			Females	Intersexes	Males	
1	<i>F^D/man</i> ⁺	<i>man/man</i>	459	0	0	0
2	<i>man</i> ⁺ / <i>man</i> ⁺	<i>man/man</i>	>2000	0	0	0
3	<i>F^D/man</i>	<i>man</i> ⁺ / <i>man</i> ⁺	944	7	0	0.7
4	<i>man</i> ⁺ / <i>man</i>	<i>man/man</i>	382	92	147	38.5
5	<i>man</i> ⁺ / <i>man</i>	<i>man</i> ⁺ / <i>man</i> ⁺	325	36	6	11.4
6	<i>M^{III}/+</i> ; <i>F^D/man</i>	<i>man</i> ⁺ / <i>man</i> ⁺	298	28	0	8.5
7	<i>M^{III}/+</i> ; <i>F^D/man</i> ⁺	<i>man/man</i>	281	71	0	20.2
8	<i>M^{III}/+</i> ; <i>F^D/man</i> ⁺	<i>man</i> ⁺ / <i>man</i> ⁺	167 ^b	0	0	0 ^c
9	<i>M^I/+</i> ; <i>M^{III}/+</i> ; <i>F^D/man</i> ⁺	<i>man/man</i>	83	66	0	44.3
10	<i>M^I/+</i> ; <i>M^{III}/+</i> ; <i>F^D/man</i> ⁺	<i>man</i> ⁺ / <i>man</i> ⁺	76 ^b	0	0	0
11	<i>man/man</i> ^d	<i>man</i> ⁺ / <i>man</i> ⁺	0	0	38	100
12	<i>M^{III}/+</i> ^d	<i>man</i> ⁺ / <i>man</i> ⁺	0	0	144 ^b	100 ^c

^a Complete genotypes see MATERIALS AND METHODS.

^b Genotype of offspring +/+.

^c HILFIKER-KLEINER *et al.* (1994).

^d Transplantation of pole cells.

to *man*⁺ males than *F^D/man* females (series 3). Similarly, only females were obtained from *F^D/man*⁺ females crossed with homozygous *man/man* males (Table 4, series 1), whereas *M^{III}/+*; *F^D/man*⁺ females (Table 4, series 7) crossed to the same males produced intersexes among their *man/+* offspring. The number of intersexual offspring further increased when the mother carried two *M* factors (*M^I/+*; *M^{III}/+*; *F^D/man*⁺; Table 4, series 9). The intersexes of all series were morphologically female with small male patches.

Homozygous *man/man* males ($n = 28$), which derived from trans-heterozygous *F^D/man* females, are sterile while the same genotype is fertile ($n > 50$) when derived from *man*⁺/*man* mothers. The introduction of an *M* factor from the father restores the fertility of the males (*M/+*; *man/man*, $n = 22$). On the other hand, fertility of the *man/man* offspring ($n > 30$) is not restored when *M^{III}* is only present in the mother (maternal genotype *M^{III}/+*; *F^D/man*). This shows that maternal *F^D* somehow interferes with fertility of NOM males.

Clonal analysis: Female larvae, heterozygous for *bwb* on chromosome III and for *man* on chromosome IV, were irradiated at different times of development to generate clones of mutant cells. Homozygous *bwb* clones on the wings, recognizable as yellow patches in a phenotypically wild-type background, provide an internal control showing that mitotic recombination, unrelated to sex, did occur. Clones with male structures in the postabdomen could be found at all times of irradiation (Table 5, Figure 1A). In contrast to the *bwb* clones, their frequencies decreased when generated later in development. The same observation was also made by WIESCHAUS and NÖTHIGER (1982) in their analysis of *tra* clones in *Drosophila*. The phenomenon

is best explained by perdurance of the wild-type product that can still exert its function if mitotic recombination has removed the wild-type allele only a few cell divisions before differentiation sets in (GARCIA-BELLIDO and MERRIAM 1971).

No clones, neither male nor *bwb*, were observed in nonirradiated sisters. Similarly, irradiation of heterozygous *bwb/+* and standard +/+ larvae produced no male clones.

Homozygous *man/man* males produce yolk proteins: Morphological differences between males and females are visible in the last five segments of the abdomen and in the interocular distance. The intersexes in the *man* stock are mosaics that consist of female and male patches side by side (Figure 1, B and C), similar to the intersexual offspring of *Ag/+* mothers (VANOSSI ESTE and ROVATI 1982; R. SCHMIDT, unpublished results). To see if morphologically normal and even fertile males from our *man* stock can also show female traits in other tissues, we looked for presence of yolk proteins in the haemolymph on Western blots with polyclonal antibodies against yolk proteins of *M. domestica*.

Of 34 homozygous *man/man* males from our stock, 13 contained yolk proteins in their haemolymph (Figure 2, lanes 3–12), and three of them produced offspring when crossed to standard wild-type females. The intersexes with external mosaicism always had yolk proteins (Figure 2, lanes 13–16). Of the heterozygous *man/+* males, four of 12 contained yolk proteins in their haemolymph (Figure 2, lanes 17–21). Interestingly, no yolk proteins were detected in the haemolymph of *man/+* males ($n = 18$) when these derived from mothers with transplanted *man/man* pole cells. In this respect, these males completely resemble wild-type males that never produced yolk proteins.

TABLE 5
Frequencies of male clones in the postabdomina, and of *bwb* clones on the wings

Genotype	Irradiated at (hr after oviposition)	Frequencies (%)	
		<i>bwb</i> clones	Male clones
<i>bwb</i> /+; <i>man</i> /+	48	8.1 (<i>n</i> = 136)	20.6 (<i>m</i> = 494)
<i>bwb</i> /+; <i>man</i> /+	72	11.0 (<i>n</i> = 82)	14.4 (<i>m</i> = 319)
<i>bwb</i> /+; <i>man</i> /+	96	90.9 (<i>n</i> = 55)	9.5 (<i>m</i> = 476)
<i>bwb</i> /+; <i>man</i> /+	120	145.5 (<i>n</i> = 66)	4.5 (<i>m</i> = 486)
<i>bwb</i> /+; +/+	48	1.5 (<i>n</i> = 66)	0 (<i>m</i> = 33)
<i>bwb</i> /+; +/+	72	7.8 (<i>n</i> = 152)	0 (<i>m</i> = 76)
<i>bwb</i> /+; +/+	96	30.3 (<i>n</i> = 60)	0 (<i>m</i> = 30)
<i>bwb</i> /+; +/+	120	131.2 (<i>n</i> = 112)	0 (<i>m</i> = 56)
+/+; +/+	72	0 (<i>n</i> = 63)	0 (<i>m</i> = 32)
+/+; +/+	96	0 (<i>n</i> = 49)	0 (<i>m</i> = 25)
<i>bwb</i> /+; <i>man</i> /+	Nonirradiated	0 (<i>n</i> = 40)	0 (<i>m</i> = 400)
<i>bwb</i> /+; +/+	Nonirradiated	0 (<i>n</i> = 56)	0 (<i>m</i> = 28)
+/+; +/+	Nonirradiated	0 (<i>n</i> = 130)	0 (<i>m</i> = 65)

n, number of wings examined; *m*, number of animals examined.

Females show at least two yolk protein isoforms (ADAMS and NELSON 1969; ADAMS and FILIPI 1983; DE BIANCHI *et al.* 1985; ADAMS *et al.* 1989), whereas in males, only the 40-kD isoform is prominent. The variability in haemolymph sampling does not permit conclusions as to the variability between individuals, but males have certainly lower titers of yolk proteins than females.

DISCUSSION

***man* has properties of an allele of *F*:** We have identified a new mutation, *man*, that affects female development: homozygous mutant animals develop as fertile males even if devoid of any male-determining *M* factor. The male pathway is also chosen by cells that become homozygous for *man* after mitotic recombination, showing that *man*⁺ function is needed for female differentiation until late in development. A similar result was found by HILFIKER-KLEINER *et al.* (1994) for the dominant female-determining factor *F*^D: clones generated in *M/M*; *F*^D/+ female larvae differentiated male structures, in accordance with their male genotype (*M/M*; +/+), even when *F*^D was removed late in development. These authors have further shown that zygotes of standard female genotype develop as males if the germ line of their mother was *M*/+, but develop as females if the mother in addition carried *F*^D (*M*/+; *F*^D/+). Thus, they

proposed that *F* must be expressed during oogenesis and that maternal *F* product is a prerequisite for the zygotic *F* gene to become active. As in the soma, *M* in the germ line is able to prevent maternal *F* function and hence has a male-determining maternal effect that is overcome by the introduction of a constitutive *F* (*F*^D) either in the germ line or in the zygote.

The described model makes two predictions for a null mutation of *F*: (1) since *F*⁺ function is necessary for female development, homozygous *F*⁻/*F*⁻ mutant animals should develop as males, and homozygous *F*⁻/*F*⁻ clones induced at any stage of development should enter the male pathway; and (2) homozygous *F*⁻/*F*⁻ pole cells, transplanted into a wild-type female host, should give rise to exclusively male offspring. Our results show that *man* fulfils these criteria. Since it also maps to the same region as *F*^D, it has the genetic properties of an allele of *F*. No recombination was observed between *man* and *Ba*, a marker located <0.5 map units from *F*^D (McDONALD *et al.* 1978). The slight difference could be due either to *man* being a small inversion with one of the breakpoints affecting the function of *F*, or to a deletion removing *F* but affecting no vital gene. Alternatively, *man* may affect a separate gene in close vicinity to *F*.

Since some of the homozygous *man/man* males produce yolk proteins, a clear female trait, *man* may not represent a complete null allele of *F* but rather a strong hypomorph. However, *man* could also be an antimorphic allele interfering with the wild-type product. Since neither a deficiency nor a duplication is available we cannot yet determine the genetic nature of the *man* allele.

A recessive mutation, *transformer* (*tra*), of *M. domestica* was described by INOUE and HIROYOSHI (1986). It was localized 0.5 map units from *Ba*, to the region of *F*^D. Homozygous *tra/tra* animals were females when they de-

FIGURE 2.—Yolk proteins in the haemolymph of females, intersexes and males derived from *man*/+ females. Lane 1, wild-type male (XY); lane 2, wild-type female (XX); lanes 3–12, *man/man* males; lanes 13–16: heterozygous *man*/+ intersexes, lanes 17–21, heterozygous *man*/+ males. Males of lanes 11, 17 and 18 were fertile.

rived from *tra*/+ mothers, but intersexes or males when they derived from *tra*/*tra* mothers. The similar properties of *tra* and *man* (localization, maternal effect) suggest that they are alleles, probably of *F*, with *tra* representing a weak and *man* a strong hypomorphic allele. Unfortunately, a complementation test with *man* and *tra* could not be done since *tra* is no longer available. We shall continue to use the designation *man* until the relation of *man* and *F* will be clarified by genetic or molecular analyses. For the following considerations, we assume that *man* is a strong hypomorphic allele of *F*.

Maternal *F* product is required for zygotic *F* activity:

Homozygous *man/man* pole cells transplanted into female embryos are capable of forming eggs, which indicates that *F* function is not needed for the development of female germ cells. On the other hand, maternal *F* function is required to initiate female development in the zygote. Eggs derived from homozygous *man/man* pole cells lack sufficient maternal *F*⁺ function to activate the zygotic *F* gene and, therefore, give rise to fertile NOM males (males without *M*). The fact that heterozygous *man*/+ mothers produce intersexual and male offspring is suggestive of a haplo-insufficiency of *man* in the female germ line. In embryos derived from such mothers, some cells may have obtained enough maternal *F* product to activate their zygotic *F* gene and to enter the female pathway; in others, the level of maternal *F* product was too low and the cells responded by entering the male pathway.

The maternal effect of *man* suggests that the sexual pathway, male or female, is chosen early in embryogenesis. The mosaic character of the intersexes in which male and female areas can extend over more than one segment (R.S., unpublished results) further delimits this phase before or at about the time when the segmental decisions are taken. Thus, sex determination in *M. domestica* and *D. melanogaster* may occur at the same time, namely around blastoderm formation.

The maternal effect of *man* is also dependent on temperature. Consistent with the effect being maternal, the temperature-sensitive phase is mainly during oogenesis. At higher temperature, more intersexes and males among the offspring of *man*/+ females are obtained, suggesting that the maternal *F* product is thermolabile. At the restrictive temperature, this results in a lower probability of activation of the zygotic *F* gene.

Since heterozygous *man*⁺/*man* females produce intersexes and males, whereas almost all of the *man*/+ offspring of *F*^D/*man* mothers develop as females (Table 4, series 5 and 3), we conclude that the masculinizing maternal effect of *man* is partially rescued by maternal *F*^D. These results suggest that more maternal *F* product is made by *F*^D than by *F*⁺, which would identify *F*^D as an overproducer. On the other hand, the rescuing activity of *F*^D is diminished by *M* factors present in the maternal genome, and the degree of this effect is proportional to the number of *M* factors (compare the

frequencies of intersexes and males in series 1, 7 and 9 of Table 4). This interaction of *M* and *F*^D in the female germ line, however, becomes only manifest when the father is homozygous *man/man* and does not contribute a wild-type allele (compare *e.g.*, series 9 and 10). Our results show that *man*/+ zygotes develop as females, intersexes or even males, depending on the genotype of their mother, and they suggest that the activity of the zygotic *F*⁺ allele is proportional to the amount of maternal products. This leads to the prediction that increasing the number of *M* factors in the germ line, *e.g.*, *M*^I/+; *M*^{II}/+; *M*^{III}/+; *F*^D/+ females, should further reduce the amount of maternal *F* product so that now all *man*/+ offspring will develop as intersexes and males, and even +/+ offspring of such females might be forced to enter the male pathway.

F^D has been considered a constitutive mutation that is epistatic to even several *M* factors. This view may still hold for somatic cells since *F*^D animals with three *M* factors are normal females (RUBINI and FRANCO 1965; RUBINI *et al.* 1972; INOUE and HIROYOSHI 1984). In the germ line, however, *F*^D seems to be more sensitive to *M* factors, suggesting that *F*^D may be differently regulated in soma and germ line.

Yolk protein synthesis is under the control of *F*:

About a third of the *man/man* males, when derived from *man*/+ mothers, synthesized yolk proteins (YPs) whereas no YPs were detected in the *man*/+ males derived from *man/man* pole cells. These results, paradoxical with respect to the zygotic genotype, identify *man* as a hypomorphic allele and emphasize the dominating role of the maternal gene product. Homozygous *man/man* males seem to have low levels of zygotic gene product, just enough to allow some YP synthesis to occur, but only when the mother carried a *man*⁺ (*F*⁺) allele that guarantees enough maternal product to activate the hypomorphic zygotic *man* alleles. In contrast, *man/man* germ cells provide insufficient amounts of maternal gene products so that the zygotic genes cannot be activated, and even *man*/+ zygotes develop as perfect, fertile males whose fat bodies do not synthesize YPs.

The results show that YP synthesis depends on the presence of *man* (*F*) gene products in the zygote. How does this product regulate YP synthesis? In *Drosophila*, the *yp* genes in the fat body cells are directly and continuously controlled in a cell-autonomous manner by *dsx*, the last member in the cascade of sex-determining genes (BOWNES *et al.* 1990; BURTIS *et al.* 1991; COSCHIGANO and WENSINK 1993). In *Musca*, *man* (*F*) appears to exert its control in a nonautonomous way by regulating hormone levels. The titer of 20-hydroxyecdysone in the haemolymph is much higher in females than in males, and it fluctuates in parallel to egg production cycles (ADAMS and FILIPI 1983; BLOMQUIST *et al.* 1984; AGUI *et al.* 1985; DE BIANCHI *et al.* 1985). YP synthesis thus is cyclical, depending on the levels of 20-hydroxyecdysone

TABLE 6

Requirement of sex-determining genes for female development in *D. melanogaster*, *C. rufifacies* and *M. domestica*

Species	Genes	Maternally required for sex determination	Zygotically required for sex determination	Required for oogenesis	References
<i>D. melanogaster</i>	<i>da</i>	Yes	No	No	CLINE (1976); CRONMILLER and CLINE (1987)
	<i>Sxl</i>	No	Yes	Yes	SÁNCHEZ and NÖTHIGER (1982); SCHÜPBACH (1985); STEINMANN-ZWICKY <i>et al.</i> (1989); CLINE (1993)
	<i>tra</i>	No	Yes	No	BAKER and RIDGE (1980); WIESCHAUS and NÖTHIGER (1982); MARSH and WIESCHAUS (1978)
<i>C. rufifacies</i>	<i>F'</i>	Yes	No	No	ULLERICH (1984)
<i>M. domestica</i>	<i>F</i>	Yes	Yes	No	HILFIKER-KLEINER <i>et al.</i> (1994); this work

and juvenile hormone. If females are hormonally deprived, *i.e.*, with ovaries, corpora allata and corpora cardiaca removed, and are then injected with a juvenile hormone analogue and 20-hydroxyecdysone, their fat bodies produce similar amounts of YPs as do those of males injected with the same dose of hormones (ADAMS and FILIPI 1988; ADAMS *et al.* 1989). This shows that the fat bodies of both sexes are competent to respond to hormonal signals, and thus, that the differential activity of *man* (*F*) in females and males does not control this competence, but rather the production or release of the necessary hormones.

Comparison of *Musca* with *Drosophila* and *Chrysomya*: NÖTHIGER and STEINMANN-ZWICKY (1985) have postulated that the different mechanisms of sex determination observed in dipteran insects may reflect a common strategy: With the help of a maternal component, an early acting primary signal in the embryo regulates a key gene whose state of activity then dictates female or male development.

The genetic control of sexual development is best known for *D. melanogaster*. In this species, the X:A ratio represents the primary signal that controls the activity of the key gene *Sex-lethal* (*Sxl*; for review see BELOTE 1992; CLINE 1993; PARKHURST and MENEELY 1994). In the presence of the maternal product of *daughterless* (*da*), *Sxl* is activated in XX; AA (female) zygotes early in embryogenesis. In X; AA (male) zygotes, it stays inactive. This state of activity, ON in females and OFF in males, must then be maintained throughout development. *Sxl* has two direct downstream target genes, namely *transformer* (*tra*) for sexual differentiation (SOSNOWSKI *et al.* 1989), and *male-specific lethal-2* (*msl-2*) for dosage compensation (KELLEY *et al.* 1995; RASTELLI *et al.* 1995; ZHOU *et al.* 1995). The requirements for the sex-determining genes during various phases of development have been determined mainly through analyses of genetic mosaics and are summarized in Table 6. The sex-determining function of *da* is purely maternal whereas the functions of *Sxl* and *tra* are purely zygotic, although *Sxl* is also needed for oogenesis.

A purely maternal mode of sex determination exists in the blowfly *C. rufifacies* where two types of females occur. Heterozygous *F'/ff* females produce only daughters, and *fff* females produce only sons (ULLERICH 1984). Thus, presence or absence of *F* in the female germ line determines the sex of the offspring.

How do *Musca* and *Chrysomya* compare with *Drosophila*? A situation paralleling that of *Chrysomya* exists in *D. melanogaster* when females are homozygous for a hypomorphic allele of *da*. Such females produce only sons; in contrast to *Chrysomya*, however, this is achieved because XX (female) zygotes are unable to activate *Sxl* and die due to failures in dosage compensation. The functional homologies of the two systems may extend to the molecular level: CLAUSEN and ULLERICH (1990) found that a probe of the *Drosophila* gene *da* hybridizes in the region of the chromosome where *F* of *Chrysomya* is located.

In *M. domestica*, previous results by HILFIKER-KLEINER *et al.* (1993) suggested that the primary zygotic signal is given by *M*, which negatively regulates the gene *F*. As shown in Table 6, *F* shares properties with *F'* of *Chrysomya* and with all three *Drosophila* genes, but also differs in important aspects from them. Its maternal effect is reminiscent of *F'* and *da*, but is not shared by *Sxl* or *tra*. Its zygotic requirement, on the other hand, is reminiscent of *Sxl* and *tra*, but contrasts with *F'* and *da*. These comparisons identify *F* as a gene for which no homologous gene has been found in *Drosophila* or *Chrysomya*. We do not yet know if *Musca* has a gene homologous to *da*, as *Chrysomya* appears to have, and if such a gene would have sex-determining functions.

A gene homologous to *Sxl* of *Drosophila* has recently been isolated in *M. domestica*. In contrast to *Sxl* of *Drosophila*, however, this gene (*SxlMd*) is ubiquitously expressed not only in females, but also in males (M. MEISE, D. BOPP and D. HILFIKER-KLEINER, personal communication), and its proteins are also found in homozygous *man/man* animals (R. SCHMIDT, unpublished results). These results show that *SxlMd* expression is independent of *man* (*F*), and that therefore a sex-determining

function of *SxlMd* is unlikely. At the present stage of analysis, it thus seems that the parallels between the sex-determining mechanisms in dipteran insects are more at the level of functional analogy rather than at the level of molecular homology.

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