The Y-Chromosomal and Autosomal Male-Determining *M* Factors of *Musca domestica* Are Equivalent

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ABSTRACT

In *Musca domestica*, male sex is determined by a dominant factor, *M*, located either on the Y, the X or on an autosome. *M* prevents the activity of the female-determining gene *F*. In the absence of *M*, *F* becomes active and dictates female development. The various *M* factors may represent translocated copies of an ancestral Y-chromosomal *M*. Double mutants and germ line chimeras show that *M^v*, *M^l*, *M^{ll}*, *M^{lll}* and *M^v* perform equivalent functions. When brought into the female germ line, they predetermine male development of the offspring. This maternal effect is overruled by the dominant female-determining factor *F^D*. *M^l* and *M^{ll}* are weak *M* factors, as demonstrated by the presence of yolk proteins in *M^l/+* males and by the occurrence of some intersexes among the offspring that developed from transplanted *M^l/+* and *M^{ll}/+* pole cells. The arrhenogenic mutation *Ag* has its focus in the female germ line and its temperature-sensitive period during oogenesis. We propose that *M^l* and *Ag* represent allelic *M* factors that are affected in their expression. Analysis of mosaic gonads showed that in *M. domestica* the sex of the germ line is determined by inductive signals from the surrounding soma. We present a model to account for the observed phenomena.

IT has been proposed that a basic system of sex determination exists in dipteran insects (NÖTHIGER and STEINMANN-ZWICKY 1985) where a key gene is positively regulated by maternal products and negatively controlled by a zygotically acting primary signal. Presence of the primary signal leads to inactivity of the key gene, and male development is the consequence. Absence of the primary signal allows the key gene to become active, leading to female development. In *Musca domestica*, several mechanisms for sex determination have been found that appear to represent variations of this basic system. A dominant male-determining factor *M* was suggested to be the primary signal that represses a postulated key gene *F* (NÖTHIGER and STEINMANN-ZWICKY 1985; INOUE and HIROYOSHI 1986; DÜBENDORFER *et al.* 1992). In standard strains, maleness is determined by the Y chromosome that carries the *M* factor (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 and HIROYOSHI 1984; INOUE *et al.* 1986). In these strains, the primary signal *M*, whether located on the sex chromosomes or on the autosomes, functions to prevent the activity of *F*.

New sex determination mechanisms might be created by mutations in *F*. A constitutive mutation would lead to female development by rendering *F* insensitive to repression by *M* and independent of the maternal prod-

ucts. This situation seems to be realized in strains where both females and males are homozygous for *M*, and female development is the consequence of a dominant factor *F^{Dominant}* (*F^D*) that is epistatic to one or several *M* factors (RUBINI 1967; McDONALD *et al.* 1978; DÜBENDORFER *et al.* 1992). Another mechanism of sex determination could result from an amorphic mutation in the *F* gene. The recessive mutation *masculinizer* (*man*) has the expected properties of such a mutation: it transforms genotypically female animals into males and maps to the position of *F* (SCHMIDT *et al.* 1997). Homozygous *man/man* animals develop as males, whereas heterozygous *man/+* zygotes enter the female pathway. In these strains, the male pathway is chosen by *man/man* animals in the absence of the primary signal *M*.

Variations in the amount of maternal products could also give rise to new mechanisms of sex determination. Zygotic *F* activity appears to require the presence of maternally synthesized *F* product, so that male development ensues if maternal *F* activity is inhibited (HILFIKER-KLEINER *et al.* 1994; SCHMIDT *et al.* 1997). This appears to be the case when *M^{lll}* is introduced into the female germ line: genotypically female offspring deriving from these germ cells become males (HILFIKER-KLEINER *et al.* 1994). Similarly, the mutation *man* shows a maternal effect leading to male development of genotypically female offspring that derived from transplanted *man/man* pole cells. This suggested that in these eggs the amount of maternal *F* product is reduced such that the zygotic *F* gene cannot become active, and these zygotes enter the male pathway (SCHMIDT *et al.* 1997). Another example for sex determination by a maternal effect is given

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by the dominant mutation *Arrhenogenic* (*Ag*). Genotypically female offspring of *Ag*/+ mothers develop as males or intersexes (VANOSSI ESTE and ROVATI 1982).

Several authors (HIROYOSHI 1964; GREEN 1980; NÖTHIGER and STEINMANN-ZWICKY 1985) have proposed that the autosomal *M* factors represent translocated copies of an ancestral *M* factor on the *Y* chromosome. Consistent with this hypothesis, *F^D* has been found to be epistatic to all of the known *M* factors tested so far (INOUE and HIROYOSHI 1982, 1984; INOUE *et al.* 1983, 1986). Based on rough mapping data, the same authors supposed that the *M* factors were not randomly inserted on the chromosomes, but are preferentially located in or near the centromeric heterochromatin.

To draw a valid conclusion, it is imperative to learn if the unexpected maternal effect observed in transplanted pole cells is unique for *M^{III}*, or is a general property of *M* factors. We thus decided to compare *M^I*, *M^I*, *M^{II}*, *M^{III}*, *M^V* and *Ag*. We could show that *M^I*, *M^{II}* and *M^V* are equivalent with respect to their function in soma and germ line, but *M^I* and *M^{II}* seem to be weaker *M* factors. *Ag* appears as an *M* that has lost the somatic function, but retained nearly full maternal activity in the germ line.

MATERIALS AND METHODS

Rearing of flies: Flies were kept as described by HILFIKER-KLEINER *et al.* (1994). For single pair crosses, flies were put 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, and *ocra* (*ochre eyes*), *apt* (*apterous*) and *snp* (*snip wings*, kindly provided by J. G. SCOTT) on linkage group V. A superscript (*X*, *Y* or roman number) is used to specify the linkage group of the *M* factors, e.g., *M^{III}* for the male determiner on linkage group III.

Table 1 lists the three strains with standard XX-XY sex determination (strains 1, 2, 3) and 11 strains with autosomal sex determination (strains 4–14) used in our experiments.

Temperature-sensitivity of *Ag*: Adult flies of the *Ag* stock were kept on 20°, 25° and 29°, respectively. Their eggs were either left to develop at the same temperature or shifted to 20° or 29° either at 0–2 hr after oviposition or in the third larval instar. Since the time to reach the third larval instar depends on the temperature, larvae grown at 29° were shifted at 72 hr after oviposition and larvae grown at 20° were shifted at 144 hr after oviposition. The frequency of males and intersexes among the offspring was determined.

Analysis of yolk proteins: Adult flies were fed for 5 days with sugar water and milk powder. At the sixth day, ~1 µl of haemolymph was taken from a slightly CO₂-anesthetized male. We used a polyclonal antibody directed against yolk proteins of *M. domestica* (1:1000 dilution in TBS/0.05% Tween-20), kindly provided by Dr. T. ADAMS, Fargo, USA. 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 as described by HILFIKER-KLEINER *et al.* (1994), except that porcine faeces were used as medium for rearing the operated animals. This allowed much better survival than the standard medium (SCHMIDT and BÄCHLI 1996) and made the use of helper larvae unnecessary. Cultures were kept at 23°. The genotypes of donor and host embryos as well as the partners used for outcrossing the host flies are specified in Table 1.

Combinations of different *M* factors with *F^D*: A cross of XX; *ac*; *M^{III}* *pw*⁺; *F^D* *Ba*/+ females and XX; *M^I* *ac*⁺/*ac*; *pw*; +/+ males produced (1) XX; *M^I* *ac*⁺/*ac*; *M^{III}* *pw*⁺/*pw*; +/+ males that were tested for presence of yolk proteins in their haemolymph and (2) XX; *M^I* *ac*⁺/*ac*; *M^{III}* *pw*⁺/*pw*; *F^D* *Ba*/+ females that were backcrossed to their fathers. Number and sex of F₂ offspring were determined. F₂ daughters of the genotype XX; *M^I* *ac*⁺/*ac*; *pw*; *F^D* *Ba*/+ or XX; *M^I* *ac*⁺/*M^I* *ac*⁺; *pw*; *F^D* *Ba*/+ were crossed individually to XX; *ac*; *ar*; *bwb* *ge* *M^{III}* *pw*⁺/*bwb* *ge* *pw*; +/+ males (strain 7, see Table 1), and number and sex of the offspring were determined. Occurrence of *Ba*⁺ females among the offspring indicated that the mother was heterozygous for *M^I*.

Females of the genotype 1, XX; *bwb*; *F^D* *Ba*/+; *M^V* *ocra*⁺/*ocra*, and 2, XX; *M^{III}* *bwb*⁺/*bwb*; *F^D* *Ba*/+; *M^V* *ocra*⁺/*ocra*, were constructed by crossing XX; *ar*; *bwb*; *F^D* *Ba*/+; *M^V* *ocra*⁺ females to XX; *M^{III}* *bwb*⁺/*bwb*; *ocra* males. For the experiment, type 1 females were backcrossed to their fathers, whereas type 2 females were crossed to *bwb*; *M^V* *ocra*⁺/*ocra* males. Number and sex of F₂ offspring of both crosses were determined.

To construct females of the genotype XX; *M^{II}* *ar*⁺/*ar*; *bwb*; *F^D* *Ba*/+, we crossed XX; *ar*⁺; *M^{III}* *pw*⁺ *bwb*/*pw* *bwb* *F^D* *Ba*/+ females to XX; *ac*; *ar*; *M^{III}* *pw*⁺ *bwb*/*pw* *bwb* males. The F₁ females were then backcrossed to their fathers. The F₂ females of genotype XX; *ar*; *pw* *bwb*; *F^D* *Ba*/+ were crossed to XX; *M^{II}* *ar*⁺/*ar*; *pw*⁺ *bwb* males.

Histology of gonads: Freshly eclosed animals were fed with milk powder and sugar water for 3 days. For dissection they were anesthetized with CO₂ and transferred into Ringer's solution. Fixation, semi-thin sectioning (1 µm) and staining of the gonads were done according to the protocol of BASLER *et al.* (1991).

RESULTS

All *M* factors have a male-determining maternal effect: It has been shown earlier that *M^{III}* exerts a maternal effect when *M^{III}*/+ pole cells were transplanted into female hosts: embryos with a female genotype became predetermined to develop as males (HILFIKER-KLEINER *et al.* 1994). To test whether *M* factors located on other chromosomes have similar maternal effects, we transplanted pole cells that were heterozygous either for *M^I* (*XY*), *M^I*, *M^{II}* or *M^V* into wild-type host embryos.

In the series with *M^I*, 52 (25.5%) host embryos survived to adulthood. Of these, 20 females and 18 males were crossed to wild-type partners. The results of the nine animals that had donor-derived progeny are given in Table 2 (series 1). XY pole cells transplanted into female hosts formed eggs all of which developed into males. We obtained 75 such males and tested 12 of them for their genotype by crossing them individually to wild-type females (XX). Four males produced daughters and sons indicating that their genotype was XY.

TABLE 1

Genetic specification of strains 1–14 and of the embryos and outcrossing partners used in the pole cell transplantation (series 1–5)

Strains				
(1) XX-XY; +/+	(6) XX; M ^{II} ar ⁺ /ar; bw <i>b</i>	(11) XX; ar; bw <i>b</i> ; F ^D Ba/+; M ^V ocra ⁺		
(2) XX-XY; ac; ar; bw <i>b</i> ; ye; sn <i>p</i>	(7) XX; ac; ar; bw <i>b</i> ge pw ⁺ M ^{III} /bw <i>b</i> ge pw	(12) XX; Ag/+ ^b		
(3) XX-XY; ac; ar; bw <i>b</i> ; ocra	(8) XX; bw <i>b</i> ⁺ M ^{III} /bw <i>b</i> ; ocra	(13) XX; Ag/+; bw <i>b</i> ; ocra ^b		
(4) XX; M ^I ac ⁺ /ac	(9) XX; bw <i>b</i> ; M ^V ocra ⁺ /ocra ^a	(14) XX; Ag/+; ar; bw <i>b</i> ^b		
(5) XX; M ^I ac ⁺ /ac; bw <i>b</i> pw	(10) XX; bw <i>b</i> ge M ^{III} /bw <i>b</i> ge M ^{III} ; F ^D Ba/+			

Transplantation series	Genotypes of donor embryos	Genotypes of host embryos	Adult hosts were crossed to	Phenotype of donor-derived progeny
1	XX; ac; ar; bw <i>b</i> ; ye; sn <i>p</i> XY; ac; ar; bw <i>b</i> ; ye; sn <i>p</i>	♀ XX; +/+ ♂ XY; +/+	♂ XY; ac; ar; bw <i>b</i> ; ye; sn <i>p</i> ♀ XX; ac; ar; bw <i>b</i> ; ye; sn <i>p</i>	ac; ar; bw <i>b</i> ; ye; sn <i>p</i>
2	XX; ac/ac; bw <i>b</i> ⁺ XX; M ^I ac ⁺ /ac; bw <i>b</i> ⁺	♀ XX; ac; ar; bw <i>b</i> ; ye; sn <i>p</i> ♂ XY; ac; ar; bw <i>b</i> ; ye; sn <i>p</i>	♂ XX; M ^I ac ⁺ /ac; bw <i>b</i> ♀ XX; ac; ar; bw <i>b</i> ; ye; sn <i>p</i>	bw <i>b</i> ⁺
3	XX; ar; bw <i>b</i> XX; M ^{II} ar ⁺ /ar; bw <i>b</i>	♀ XX; +/+ ♂ XY; +/+	♂ XX; +/+; ar; bw <i>b</i> ^f ♂ XX; Ag/+; ar; bw <i>b</i> ^f ♀ XX; ac; ar; bw <i>b</i> ; ye; sn <i>p</i>	bw <i>b</i> ^f
4	XX; bw <i>b</i> ; ocra XX; bw <i>b</i> ; M ^V ocra ⁺ /ocra	♀ XX; +/+ ♂ XY; +/+	♂ XX; +/+; bw <i>b</i> ; ocra ^f ♂ XX; Ag/+; bw <i>b</i> ; ocra ^f ♀ XX; ac; ar; bw <i>b</i> ; ocra	bw <i>b</i> ^d
5a	XX; Ag/+; bw <i>b</i> ; ocra XX; +/+; bw <i>b</i> ; ocra	♀ XX; +/+ ♂ XY; +/+ ^f	♂ XX; M ^{III} bw <i>b</i> ⁺ /bw <i>b</i> ; ocra	ocra
5b	XX; Ag/+; bw <i>b</i> ; ocra XX; +/+; bw <i>b</i> ; ocra	♀ XX; +/+ ^g	♂ XX; M ^{III} bw <i>b</i> ⁺ /bw <i>b</i> ; ocra	ocra

^a The M^V chromosome was isolated from a wild strain from Turkey kindly provided by Dr. ŞÜKRAN ÇAKIR.

^b Two types of females exist in the Ag-strains: (1) females of the genotype XX; Ag/+ that are arrhenogenic, i.e., produce NOM-males (males with no M) and intersexes, and (2) females of the genotype XX; +/+ that are thelygenic, i.e., produce only daughters (VANOSSI ESTE and ROVATI 1982).

^c Among these, animals carrying no M^H showed the ar phenotype (except very rare recombinants; map distance 0.4 cM).

^d Among these, animals with no M^V had ocra eyes (except for 10% recombinants).

^e NOM males of stock (13).

^f These male hosts were discarded.

^g These females were produced by crossing XX; +/+ females (stock 1) to NOM males of the genotype XX; Ag/+ or XX; +/+ (stock 13).

Five males produced only sons indicating that they carried two Y chromosomes. Finally, three males produced only females, showing that they carried no Y chromosome and therefore were NOM males (males with no M). Obviously, M^I had been active in premeiotic stages, and its activity was sufficient to direct the development of genotypically female zygotes into the male pathway.

In the series with M^V , 104 (28.6%) injected wild-type host embryos survived to adulthood. Of the 19 females that were successfully crossed to NOM males, seven produced donor-derived sons (eight or more), but no donor-derived daughters. This indicates that these females had integrated donor pole cells of the genotype M^V /+. To determine the genotype of their male offspring, we crossed 48 of the homozygous ocra males individually to wild-type females (XX). Five males generated sons and daughters, indicating that they were M^V ocra/ocra recombinants, whereas 43 turned out to be NOM males that only produced daughters (Table 2, series 4). Thus, M^V shows a maternal effect equal to the one of M^I and M^{III} .

In the series with M^I , 97 (33.8%) adult flies were obtained and crossed to test partners. In contrast to the pure male offspring that resulted when M^V or M^H were introduced into the female germ line, males, intersexes and females developed from XX; M^I /+ germ cells transplanted into female hosts (Table 2, series 2). This indicates that the maternal effect of M^I is weaker than the maternal effects of M^V , M^{III} or M^H . Fifty-one males of the donor-derived offspring were crossed individually to wild-type females (XX) to define their genotype: 15 of them were shown to be NOM males that only produced daughters, whereas the rest produced daughters and sons, indicating that they were M^I /+ heterozygotes. Surprisingly, none of the tested males turned out to be homozygous for M^I , which suggests that the M^I chromosome carries a recessive lethal.

M^{III} gave a similar result: 42 (22.5%) injected host embryos developed to the adult stage, and 19 females could be successfully crossed to NOM males. Of the 14 females that were mosaic in the germline (Table 2, series 3), six had exclusively male donor-derived off-

TABLE 2

Pole cells with either M^I , M^I , M^{II} , or M^V show a male-determining maternal effect when transplanted into wild-type female hosts

Series	Genetic sex of donor pole cells	Sex of host ^a	Adult host was crossed to	No. of host-derived offspring		No. of donor-derived offspring		
				Females	Males	Females	Intersexes	Males
1	XX	XX (2)	XY	50	62	4	0	3
	XX	XY (5)	XX	190	216	166	0	0
	XY	XY (1)	XX	108	111	6	0	9
	XY	XX (1)	XY	10	11	0 ^b	0	75 ^c
2	XX; +/+	XX (11)	XX; M^I /+	208	200	54	0	58
	XX; +/+	XY (7)	XX; +/+	41	37	290	0	0
	XX; M^I /+	XY (2)	XX; +/+	34	30	81	0	59
	XX; M^I /+	XX (8)	XX; M^I /+	132	114	21	11 ^d	164 ^c
3	XX; +/+	XX (6)	NOM males ^e	839	0	266	0	0
	XX; +/+	XY (6)	XX; +/+	43	45	328	0	0
	XX; M^{II} /+	XY (7)	XX; +/+	104	92	189	0	142
	XX; M^{II} /+	XX (8)	NOM males ^e	1146	0	0	5 ^d	698 ^c
4	XX; M^V /+	XY (10)	XX; +/+	251	219	421	0	399
	XX; M^V /+	XX (7)	NOM males ^f	824	0	0	0	470 ^c

Donor- and host-derived offspring were distinguished by the presence of markers (see corresponding series in Table 1).

^a Listed are only those hosts that had donor-derived progeny; number of hosts in parentheses.

^b Although 1/4 is expected to be XX, no females were obtained.

^c Outcrosses with standard females proved the presence of males with female genotype (NOM males) in this group.

^d The intersexes were of the mosaic type.

^e The NOM males were of genotype XX; Ag /+; ar ; bwb or XX; +/+; ar ; bwb .

^f The NOM males were of genotype XX; Ag /+; bwb ; $ocra$ or XX; +/+; bwb ; $ocra$.

spring, whereas two produced mostly males but also a few intersexes (four out of 85 and one out of 24). We crossed 31 homozygous ar males to standard females (XX), and, as expected from the short mapping distance between M^{II} and ar (see Table 1, footnote c), all of them turned out to be devoid of M .

F^D is epistatic to all M factors in the germ line: The maternal effect of M^{III} is counteracted by the simultaneous presence of F^D in the female germ line such that +/+; +/+ offspring of M^{III} /+; F^D /+ mothers develop as females (HILFIKER-KLEINER *et al.* 1994). We asked whether the maternal effect of M^I , M^{II} and M^V , or even the combined masculinizing maternal effects of M^I and M^{III} , or M^{III} and M^V , can be overruled by the maternal effect of F^D .

To test for epistasis of F^D to M^I in the female germline, we constructed females of the genotype $M^I ac^+/ac$; pw ; $F^D Ba$ /+ and crossed them to ac ; $M^{III} pw^+/pw$; +/+ males. Since M^{III} is closely linked to pw^+ , and F^D is closely linked to Ba , the offspring without M^{III} and F^D could be identified by their pw ; Ba^+ phenotype. Absence or presence of M^I is not as reliably recognized, because it is separated from ac by ~34 map units (ROVATI *et al.* 1983). Among the 2243 offspring, we obtained 173 $ac pw Ba^+$ and 18 $ac^+ pw Ba^+$ females, 51 $ac pw Ba^+$ males and 279 $ac^+ pw Ba^+$ males. To test whether the $ac pw Ba^+$ males carried an M factor as a result of recombination between M and ac , we crossed them individually

to standard females. All of them produced daughters and sons, demonstrating that they were of the recombinant genotype $M^I ac/ac$; pw ; +/+. Thus, we know that there were no NOM males among the offspring, which indicates that all +/+; +/+ offspring of M^I /+; F^D /+ mothers developed as females. For unknown reasons, our data give a much smaller recombination value for M^I and ac (13 map units) than ROVATI and coworkers (1983) found.

To test whether the maternal effect of F^D is epistatic to the maternal effect of M^V , we crossed bwb ; $F^D Ba$ /+; $M^V ocra^+/ocra$ females to $M^{III} bwb^+/bwb$; $ocra$ males. Offspring without M^{III} and F^D are easily recognized by their $bwb Ba^+$ phenotype, but animals with $ocra$ eyes could be of genotype $ocra/ocra$ or $M^V ocra/ocra$ since the distance between M^V and $ocra$ is ~10 map units (INOUE and HIROYOSHI 1984). By testcrosses, we could show that all offspring without M and F^D developed as females, which indicates that the maternal effect of F^D can also counteract the maternal effect of M^V (data not shown).

A similar result was obtained for M^{II} . A cross of XX; $M^{II} ar^+/ar$; bwb ; $F^D Ba$ /+ females and XX; $M^{II} ar^+/ar$; bwb males produced 353 $ar^+ Ba^+$ males, 174 $ar^+ Ba$ females, 152 $ar Ba$ females and 279 $ar Ba^+$ females. In addition, one recombinant $ar^+ Ba^+$ female occurred. Since this phenotypic class represents one-fourth of all recombinants, the distance between M and ar is ~0.4 cM (4/

FIGURE 1.—Presence of yolk proteins in the haemolymph of heterozygous $M^I/+$ males shown by Western blotting. Lane 1, wild-type female (XX); lane 2, wild-type male (XY); lane 3, wild-type female (XX) one-third loaded; lanes 4–14, heterozygous $M^I/+$ males.

959), which corresponds to the published distance of INOUE and HIROYOSHI (1984). The results clearly show that the animals without M^I and F^D ($ar^+ Ba^+$) develop as females demonstrating that in the female germ line F^D is also epistatic to M^I .

To obtain females of the genotype $XX; M^I ac^+/ac; M^{III} pw^+/pw; F^D Ba^+/+$, we crossed $ac; M^{III} pw^+/M^{III} pw^+; F^D Ba^+/+$ females to $M^I ac^+/ac; pw$ males. The desired females were then backcrossed to their fathers. In the F_2 , we obtained, among others, 69 $ac pw Ba^+$ females and 20 $ac pw Ba^+$ males. These males were again tested for M^I by crossing them to wild-type females. All of them produced daughters and sons, indicating that they were indeed recombinants of the genotype $M^I ac/ac; pw/pw; +/+$. This showed that F^D is epistatic to the combined maternal effects of M^I and M^{III} . Similarly, when double-heterozygous $M^{III}/+; M^V/+; F^D/+$ females were crossed to $bwb; M^V ocr^+/ocr^+$ males, the offspring lacking M and F^D developed as females (data not shown).

Yolk protein synthesis in $M^I/+$ males: Since the effect of M^I is weak in the female germ line, we wondered whether the zygotic function of M^I might also be reduced. A reduction of M function would allow low levels of zygotic F product to be made that could lead to the differentiation of female traits, such as female external structures or production of yolk proteins. Close microscopic inspection of heterozygous $M^I/+$ animals revealed no morphological intersexuality in the stock. Yolk proteins, however, were present in the hemolymph of 23 out of 41 $XX; M^{II}/+$ males (Figure 1). The amount of yolk proteins was variable and much lower than in females. No yolk proteins were ever detected in wild-type XY males ($n > 40$), heterozygous $M^{II}/+$ ($n = 19$), $M^{III}/+$ ($n = 20$) and $M^V/+$ ($n = 12$) males. We also tested 22 NOM males derived from transplanted pole cells that were either of genotype $M^{II}/+$ (11 cases) or of genotype $M^V/+$ (11 cases). In none of them could we detect any yolk polypeptides. One tested intersexual NOM fly of the $M^{II}/+$ series was clearly positive, as were all the female controls.

Yolk protein synthesis is blocked in $M^I/+; M^{III}/+$ males: The production of yolk proteins by heterozygous $M^I/+$ males could be due to insufficient M function, but also to a mutation in the yolk protein gene(s)

FIGURE 2.—No yolk proteins were detected in doubly heterozygous $M^I/+; M^{III}/+$ males on Western blots. Lane 1, wild-type female (XX); lane 2, wild-type male (XY); lane 3, $XX; M^{III}/M^{III}; F^D/+$ female; lane 4, $XX; M^{III}/+$ male; lanes 5 and 6, $XX; M^I/+$ males; lanes 7–9, $XX; M^I/+; M^{III}/+$ males.

leading to their constitutive expression in males. Since $M^{III}/+$ males produce no yolk proteins, we constructed double-heterozygous $M^I/+; M^{III}/+$ males and tested them for presence of yolk proteins. None ($n = 18$) showed any yolk proteins in the haemolymph, indicating that yolk protein production in the heterozygous $M^I/+$ males is not due to a dominant regulatory mutation in the yolk protein gene(s). To rule out the possibility of a recessive mutation in the yolk protein gene(s), we backcrossed the F_1 females of the genotype $XX; M^I ac^+/ac; M^{III} pw^+/pw; F^D Ba^+/+$ to $XX; M^I ac^+/ac$ males. In the F_2 , we tested the double-heterozygous $M^I/+; M^{III}/+$ males as well as males without M^{III} for presence of yolk proteins in the haemolymph. Although heterozygous $M^I/+$ and homozygous M^I/M^I males are phenotypically indistinguishable, our observation in the pole cell experiment suggests that homozygosity for M^I is lethal. Thus, we can argue that all M^I males in this experiment are heterozygous. Of the three tested M^I males, one showed yolk proteins whereas none of the double heterozygous $M^I/+; M^{III}/+$ males ($n = 16$) did (Figure 2). We conclude that yolk protein synthesis in $M^I/+$ males is not due to a mutation in the yolk protein gene(s) but to reduced levels of M function.

Yolk protein synthesis in sons of $Ag/+$ females: VANOSSI ESTE and ROVATI (1982) described a mutation with a dominant maternal effect, *Arrhenogenic* (*Ag*). Genotypically female offspring of $Ag/+$ mothers develop as fertile males or intersexes. We suspected that cryptic intersexes might be present among the flies that externally appeared as males. We therefore tested intersexes and males derived from heterozygous $Ag/+$ females for presence of yolk proteins in the haemolymph. All visibly intersexual flies produced yolk proteins. Contrary to our expectation, however, no yolk proteins were detected in the haemolymph of 38 males, suggesting that these males were not intersexual in internal structures.

Temperature-sensitivity of Ag : When kept at 25° or 29° throughout development, most of the offspring from a mixed population of heterozygous $Ag/+$ and $+/+$ females and males developed as females, and only few animals were intersexes or males (Table 3). At 20°, many more intersexual and male offspring were obtained. Since the percentages of intersexes and males

TABLE 3
The temperature-sensitive phase of *Ag* is during oogenesis

Developmental stage of shifted animals	Temperature before and after shift (°)	Frequency of offspring (%) ^a		n
		Females	Intersexes and males	
Embryos	20/29	44.4	55.6	941
	29/20	83.4	16.6	821
Third instar	20/29	63.8	36.2	1000
	29/20	72.0	28.0	397
Controls	20/20	65.8	34.2	3166
	25/25	91.8	8.2	6899
	29/29	95.6	4.4	5862

^a Offspring of XX; *Ag*/+ and XX; +/+ females crossed to XX; *Ag*/+ and XX; +/+ males. Mothers were kept at either 20° or 29°, and offspring were shifted either 0–2 hr after oviposition (embryos) or in third instar, or left at the same temperature (controls).

among the offspring are similar at 25° and 29°, the critical temperature must lie between 20° and 25°.

The temperature-sensitive period was determined by shifting the offspring during embryonic or larval development (Table 3). Of the animals shifted from 20° to 29° immediately after oviposition or in the third larval instar, a large proportion developed as intersexes and males, mimicking the situation of continuous development at 20°. This result indicates a temperature-sensitive period during oogenesis. The data are less clear for shift-down experiments, but they point in the same direction. The frequencies of males and intersexes are somewhat elevated, but do not reach the levels of the controls kept at 20°. Since the extent of the masculinizing effect of *Ag* mainly depends on the temperature regime to which the mother was subjected before oviposition, we conclude that *Ag* exerts its major effect during oogenesis. A slight zygotic effect, however, is not ruled out by the data.

The temperature-sensitive period for *Ag* during oogenesis indicates an early effect on sexual development. In a threshold reaction to minimal levels of maternal *F* product, the zygotic *F* gene may either become active or not. The existence of intersexes that consist of a mosaic of male and female cells shows that this decision is a cellular and early event.

***Ag* acts cell-autonomously in the female germ line:** We wondered whether the masculinizing maternal effect of *Ag* has its focus in the germ line or is imposed on the germ cells by the surrounding soma. To answer the question, we transplanted pole cells from embryos of an *Ag* stock into wild-type hosts and obtained 209 (24.4%) adult females. Of the 159 females that were individually crossed to XX; +/+; *M*^{III}/+ males, 37 gave rise to donor-derived offspring. As expected, ~50% of these inherited the *M* factor from their father and therefore developed as males. The interesting class, however, are those flies that had a female genotype (+/+; Table 4). The +/+ progeny were female in 19

cases and male or intersexual in 18 cases. The former progeny must have derived from transplanted +/+ pole cells and the latter from *Ag*/+, and from this we conclude that *Ag* acts autonomously in the female germ line.

The sex of the germ cells is dictated by the gonadal soma: Transplantation of pole cells has shown that germ cells with a female genotype are able to form functional sperm in a male host, and germ cells with a male genotype form eggs in a female host (Tables 2 and 4; see also HILFIKER-KLEINER *et al.* 1994). This is consistent with the finding that YY; *F*^D/+ females, as well as males with a female genotype derived from *Ag*/+ mothers, are both fertile (RUBINI and PALENZONA 1967; VANOSSI ESTE and ROVATI 1982).

The nonautonomous development of the germ line might be imposed on the transplanted germ cells by the gonadal soma or by germ cells of the host. To discriminate between these alternatives, we analyzed sexually mosaic gonads of intersexes derived from *Ag*/+ mothers. In these gonads ovarian and testicular tissues lie side by side so that male and female signals occur in the same gonad (D. HILFIKER-KLEINER, personal communication). In semi-thin sections, the orange pigmentation of the testis is easily recognizable as a dark layer (Figure 3D), whereas the female somatic tissue is white. In testes of control males, the young stages of spermatogenesis are found close to the somatic testis sheath while elongating spermatid bundles are found in the central part (PERJE 1948). In control females, the ovary consists of several ovarioles with cysts of different stages (Figure 3C). All of these cysts, which contained nurse cells and the oocyte, were surrounded by follicle cells.

In mosaic gonads, we found oogenic germ cells associated with ovarian tissue, and spermatogenic germ cells associated with testicular tissues. Clearly male germ cells were sometimes located next to clearly female germ cells (Figure 3, A and B), which shows that cells of the female germ line did not affect the differ-

TABLE 4

Transplantation of *Ag*/+ and +/+ pole cells shows that *Ag* has its focus in the female germ line

Inferred genotype of donor pole cells	Host females ^a	Adult female was crossed to	No. of host-derived offspring (<i>ocra</i> ⁺)		No. of donor-derived offspring (<i>ocra</i>)			
			Females	Males	<i>M</i> /+ males	+/+ females	+/+ intersexes ^b	+/+ males
<i>XX; Ag</i> /+	<i>XX; +/+</i> (18)	<i>XX; +/+; M^{III}/+</i>	1117	1194	251	0	2	247
<i>XX; +/+</i>	<i>XX; +/+</i> (19)	<i>XX; +/+; M^{III}/+</i>	1133	1135	168	161	0	0

Donor- and host-derived offspring were distinguished by their eye color (for complete genotypes see Table 1, series 5a and 5b). The genotype of the donor pole cells is inferred from the presence or absence of +/+ males.

^a Values in parentheses are number of hosts.

^b The intersexes are of the mosaic type.

entiation of neighboring male germ cells, and vice versa. Germ cells that occurred at about equal distance from female and male somatic cells were sexually undifferentiated and could not be identified as oogenic or spermatogenic.

DISCUSSION

The *M* factors are functionally equivalent: Based on their male-determining effect, the various autosomal *M* factors were assumed to be translocated copies of an ancestral *M* originally located on the *Y* chromosome (HIROYOSHI 1964; GREEN 1980; NÖTHIGER and STEINMANN-ZWICKY 1985). The view of *M* being a mobile element implies that all *M* factors should be equal with respect to their function. This model received support from the fact that the dominant female-determining factor *F^D* is epistatic to all *M* factors tested so far (INOUE and HIROYOSHI 1982, 1984; INOUE *et al.* 1983, 1986). Besides that, little is known about the functional characteristics of the autosomal and the *Y*- or *X*-chromosomal *M* factors, with the exception of *M^{III}*. Clones of cells from which *M^{III}* had been removed by mitotic recombination during larval development of *M^{III}/+* males still formed male structures despite their female genotype (HILFIKER-KLEINER *et al.* 1993). This suggested that *M^{III}* suppresses the activity of the *F* gene early in development and that this state of inactivity is afterward propagated independently of *M*. The same authors also showed that *M^{III}/+* pole cells, when transplanted into female hosts, formed eggs, which indicates that *M* in the germ line does not dictate male gametogenesis. From these eggs, however, only males developed even in the absence of an *M* in the zygote, which proves that *M^{III}* is active during oogenesis and causes a male-determining maternal effect. On the other hand, embryos without *M* or *F^D*, produced by *M^{III}/+*; *F^D/+* mothers, developed into females, showing that *F^D* is epistatic to *M* in the germ line. These results were interpreted to indicate that *M^{III}* is active in the female germ line where it prevents the activity of *F*, and that maternal function of *F* is required for the zygotic *F* to become active.

Our experiments demonstrate that *M^V*, *M^I*, *M^{II}* and *M^V* have essentially the same properties as *M^{III}*: they determine male sexual development and are hypostatic to *F^D*; they negatively regulate synthesis of yolk proteins, and they have a male-determining maternal effect in the female germ line. Although the qualitative properties of these *M* factors are equal, there appear to be variations in strength, as shown by *M^I* and *M^{II}*. *M^I/+* animals are fertile males, but produce variable levels of yolk proteins. When brought into the female germ line, both *M^I* and *M^{II}* have male-determining maternal effects, but these are weaker than those of the other *M* factors, as indicated by the appearance of rare intersexual offspring.

Our results thus show that all the tested *M* factors perform the same basic function, which provides further support for the view that the *M* factors found in different strains on different chromosomes are translocated copies. Characteristics of a mobile element were also assigned to the male-determining factor identified in the phorid fly *Megaselia scalaris* Loew (MAINX 1966; TRAUT and WILLHOEFT 1990). The different levels of activity of the various *M* factors of the housefly may be the result of a positional effect. This phenomenon is well known to influence the level of expression of transgenes in *Drosophila melanogaster*. A definite answer whether the different *M* factors are indeed copies of an ancestral *M*, or are different genes that achieve the same developmental result, will come from a molecular analysis.

***Ag* may be a regulatory mutation of *M*:** The *Ag* factor may be another case of variable expression of *M*. Our experiments show that the arrhenogenic effect of *Ag*/+ mothers acts cell autonomously in transplanted pole cells, in a similar way as *M^I*. But whereas *M^I/+* animals are fertile males, *Ag*/+ animals are fertile females, indicating tissue-specific differences in the activity of these two factors.

M^I was isolated from an *Ag* stock and maps to the same region on the first chromosome as *Ag* (ROVATI *et al.* 1983). Whereas *M^I* displays only a slightly reduced activity in the soma and in the germ line, *Ag* has largely

FIGURE 3.—Semi-thin sections of mosaic gonads from intersexes derived from *Ag/+* females. (A and B) Mosaic gonad where the male somatic tissue (mS) is recognizable as a dark cell layer. fs, female somatic tissue; NC, nurse cells; S, spermatid bundles. Germ cells with a clear female morphology are located next to germ cells with male characteristics indicating that the surrounding somatic tissue directs the sexual differentiation of the germ cells. (C) Section through a wild-type ovary that is composed of several ovarioles. The cysts containing the nurse cells are surrounded by somatic follicle cells. (D) Section through a testis of a control male (XY). The somatic tissue appears as a dark cell layer. The early stages of spermatogenesis (spermatocytes) are seen on the right side while elongating spermatids are located to the middle of the testis. Bars, 100 μ m.

lost its somatic function, but retained its effect in the female germ line. In this tissue, but not in the soma, it can prevent the activity of *F*, as shown by the fact that *F^D* is epistatic to *Ag* in the germ line (ROVATI and VANNOSSI ESTE 1978), *i.e.*, prevents the effect of *Ag*. It thus appears that *M* can be subject to differential regulation in soma and germ line, and we might expect that the reverse of *Ag*, namely an *M* factor that has retained the somatic, but lost the germ line function, may also exist.

cis-regulatory elements that are differently used in soma and germ lines have been identified for *Sxl* in *D. melanogaster*. *Sxl* is the master control gene for sexual differentiation in the soma where it is transcribed from a promoter *P_e* that is activated only in females by two doses of X-linked numerator genes (CLINE 1993). *Sxl* is also required in the female germ line. In this tissue, however, *Sxl* is not transcribed from *P_e* (KEYES *et al.* 1992), and the activating numerator genes, if there are any, are not the same as those in the soma (GRANADINO *et al.* 1993; STEINMANN-ZWICKY 1993). Further parallels for differential regulation in soma and germ line are provided by *Sxl* mutations that affect either only the somatic or only the germ line function of the gene (SALZ *et al.* 1989).

The sex of the germ cells is dependent on somatic

signals: In *D. melanogaster*, autonomous and inductive signals cooperate to control the sexual differentiation of germ cells. XY and XO germ cells enter spermatogenesis even when they are embedded in female gonadal tissue, thus displaying autonomous features of sexual differentiation. On the other hand, spermatogenesis is nonautonomously initiated by XX or XXY germ cells in a male host (STEINMANN-ZWICKY *et al.* 1989; STEINMANN-ZWICKY 1992).

In more primitive insects, such as *Chrysomya rufifacies* or *M. domestica*, transplanted pole cells develop nonautonomously and form functional gametes. They complete normal oogenesis in a female host, or normal spermatogenesis in a male host, regardless of their own genetic sex (ULLERICH 1984; HILFIKER-KLEINER *et al.* 1994; this work). Since implanted pole cells are always in contact with the germ line of the host, it remained unclear whether the transplanted pole cells reacted to signals emanating from the soma or from the neighboring germ cells.

In mosaic gonads of intersexes derived from *Ag/+* females, sexual morphology of the germ cells corresponds to the sex of the surrounding somatic tissue, but not to the sex of neighboring germ cells. This indicates that the essential signals for sex determination in the

FIGURE 4.—Model for the action of *M*, *Ag* and *F* in soma (symbolized by shaded ovals) and germ line (white circles). Crossed gene symbols indicate the inactive state; parentheses mean unknown state of activity. (A) In a normal female, absence of *M* in the soma permits *F* to be active, which leads to female somatic development. As a consequence, the cells of the germ line develop into eggs. Since *F* was active in the germ line, the eggs are amphogenic, *i.e.*, both sexes can develop from them, depending on whether or not the sperm contributes an *M* factor. (B) In a normal male, *M* in the soma prevents activity of *F*, which leads to male development. As a consequence, the cells of the germ line differentiate into sperm. This is also the case if the germ line develops from transplanted pole cells that are devoid of *M*. The functional state of *M* and *F* in spermatogenesis is unknown. Since normal sperm are formed in a male host by transplanted germ cells without *M* and even by germ cells with *F^D* (HILFIKER-KLEINER *et al.* 1994), we conclude that neither of these genes plays a direct role in spermatogenesis. (C) In a female host, transplanted *M*/+ pole cells are forced into the oogenic pathway as a consequence of inductive effects of the female soma. In the transplanted pole cells, *M* is premeiotically active and prevents activity of *F*. Without maternal *F* product, the zygotic *F* cannot become active. Such eggs are arrhenogenic, *i.e.*, give rise to exclusively male offspring, irrespective of the genetic contribution by the sperm. (D) *Ag* does not repress *F* in the soma, which leads to female development of the soma and germ line. In the germ cells, however, *Ag* prevents activity of *F* with the same consequences as in C.

germ line originate from somatic cells. Germ cells at equal distances from testicular and ovarian somatic tissue seem to remain sexually undifferentiated. They may have received either no sex-determining signal or conflicting signals from both ovarian and testicular tissue.

Concluding remarks and a model: The simplest hypothesis compatible with all the data obtained so far is

that the different *M* factors have derived by transposition from an ancestral *M* whose function is to prevent the activity of the female-determining gene *F*. Normally, this repressing function is only required in the soma, but it can obviously also be performed during oogenesis in the germ line of *Ag*/+ females or in *M*/+ pole cells transplanted into female hosts.

The functional relationships, as seen today, between *M* and *F* and between soma and germ line, are illustrated in Figure 4. The essence of this model can be summarized in three statements. (1) Expression, or non-expression, of *F* in the soma determines whether female or male development will occur. (2) The sex of the gonadal soma forces the germ cells into oogenesis or spermatogenesis. (3) Expression of *F* during oogenesis leads to maternal *F* product that is required if embryos are to develop as females. This model is based on results obtained with *M. domestica*. In principle, however, it may apply to other insects as well.

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