The *Drosophila misfire* gene has an essential role in sperm activation during fertilization

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The male sterile mutation, *misfire* (*mfr*), of *Drosophila melanogaster* is a novel paternal effect, fertilization defective mutant that effects sperm head decondensation. *mfr* sperm were motile, appeared normal morphologically and were transferred to the female during copulation. However, less than 0.1% of eggs laid by females mated to *mfr* males hatched. Although *mfr* sperm entered eggs at a high frequency (93%), 99% of the inseminated eggs did not initiate the first nuclear division. Unlike wild type fertilizing sperm, the position and shape of *mfr* sperm tails within the egg were not constant, but varied in a seemingly random manner. The heads of inseminating mutant sperm were always located near the surface of eggs just underlying the egg plasma membrane, and maintained their needle-like shape indicating the failure of nuclear decondensation. Further observations revealed that plasma membrane of inseminating sperm appeared intact, including the head region. These phenotypes were equivalent to those of *sneaky* (*snky*), another fertilization defective male sterile mutation. Our observations strongly suggest that *mfr* mutant males are sterile because their inseminating sperm fail to form a male pronucleus due to the inability of the sperm to properly respond to egg factors responsible for the breakdown of the plasma membrane. Although *mfr* and *snky* mutations were phenotypically identical, they mapped to cytologically distinct genetic loci and no genetic interactions were observed, suggesting that at least two distinct paternal gene products are involved in the early stages of pronuclear formation.

Key words: male sterile, paternal effect, pronuclear formation, spermiogenesis

INTRODUCTION

In sexually reproducing organisms, fertilization, which begins with the initial contact of the egg and sperm membranes (syngamy) and ends with the union of the maternal and paternal nuclei (karyogamy), is the critical first step in the formation of a viable diploid zygote capable of successfully carrying out the developmental program. Fertilization processes include sperm-egg recognition, sperm entry into the egg cytoplasm, formation of male and female pronuclei, migration of the pronuclei, and finally fusion of the pronuclei to form diploid zygotic nuclei. These events usually proceed in rapid sequence and require precise interactions between egg and sperm.

The successful completion of fertilization and the initiation of embryogenesis require the precise coordination between maternal and paternal gene products. The maternal and paternal effect mutations, two classes of sterile mutations that produce morphologically normal gametes, and either fail to complete fertilization or result in embryonic lethality, have been studied extensively in *Drosophila melanogaster* and *Caenorhabditis elegans* (Karr, 1996; Fitch et al., 1998). Genetic and cytological studies of these mutations identify key elements of fertilization mechanisms.

In *D. melanogaster*, many mutations that affect fertilization processes and/or early mitotic cleavages have been identified and characterized. The majority of this class of mutations is maternal effect mutations (Foe et al., 1993) that identify either structural or regulatory elements needed in the egg for fertilization. Not unexpectedly, far fewer paternal effect mutations have been reported (Foe et al., 1993; Karr, 1996; Fitch et al., 1998) as the egg provides the vast majority of the components required for the fertilization and early embryogenesis. For example, maternal effect mutations, including *giant nuclei* (Freeman et al., 1986; Freeman and Glover, 1987), *pan gu* (Shamanski and Orr-Weaver, 1991), and *plutonium* (Shamanski and Orr-Weaver, 1991; Axton et al.,...
1994), affect the mitotic behavior of both the male and female pronuclei, others including *sésame* (Loppin et al., 2000, 2001b) and *maternal haploid* (Santamaria and Gans, 1980; Loppin et al., 2001a), impede specifically formation of the male pronucleus. These mutations demonstrate that the male pronucleus formation requires maternally supplied components. Although maternal effect mutations have been extensively studied and have defined many of the components necessary for the initiation and maintenance of zygotic development (Foe et al., 1993), paternal effect mutations are just beginning to be identified and studied (Karr, 1996; Fitch et al., 1998). Paternal effect mutations are defined as those that do not affect the process of spermato genesis per se, but instead affect the function of sperm during and following fertilization. The existence of this class of mutations has clearly defined a role for sperm in fertilization and zygote formation. Therefore, isolation and characterization of paternal effect mutations are important for understanding paternal contributions toward fertilization processes.

Extensive genetic screens have identified about 1,500 genes in *D. melanogaster* that when mutated result in male sterility (Hackett et al., 2000). However, of these only three paternal effect mutations on the fertilization processes have been characterized. These mutations induce three different phenotypic classes. The first class of the mutation, *casanova* (*csn*) affects sperm-egg recognition. The *csn* sperm fails to penetrate into the egg (Perotti et al., 2001). Two glycosidases, β-N-acetyl glucosaminidase and α-D-mannosidase, localized on the plasma membrane of sperm are suggested to have an important role for sperm-egg recognition (Cattaneo et al., 1997; Pasini et al., 1999). Biochemical evidence suggests that *csn* sperm lack β-N-acetylglucosaminidase on the plasma membrane covering the acrosome, suggesting that this prevents sperm from entering the egg (Perotti et al., 2001). The second, *sneaky* (*snky*) mutation affects male pronuclear formation. *snky* sperm does enter the egg but the sperm head remains near the surface of the egg and fails to form a functional male pronucleus. *snky* appears to affect sperm activation in the egg, presumably by preventing the degradation of the sperm plasma membrane (Fitch and Wakimoto, 1998). The third class of mutation is *ms(3)K81*, which affects the final step of fertilization. The *ms(3)K81* sperm penetrate the egg, form a male pronucleus but the paternal chromosomes are lost during the first mitotic division, which leads to either abortive embryogenesis or haploid development. Eggs fertilized by *ms(3)K81* sperm die before hatching (Fuyama, 1984, 1986a; Yasuda et al., 1995). The mutation potentially affects either the formation of male pronucleus or processes necessary for the first mitotic division.

Here, we describe a novel paternal effect mutation of *D. melanogaster*, *misfire* (*mfr*). Our genetic and cytological analyses of the *mfr* mutant phenotype indicate that *mfr* product is essential for completion of fertilization. The mutant phenotype is similar to *snky*, *mfr* sperm penetrates the egg but fails to form a male pronucleus. The inseminating sperm heads of *mfr* are always found in the periphery of eggs and retain needle-like morphology as the mature sperm. Our observations strongly suggest that sterility of *mfr* males is attributed to a failure of the plasma membrane to interact properly with maternal products in the egg cytoplasm in order to initiate pronuclear formation.

**MATERIALS AND METHODS**

*Drosophila* strains. All cultures were raised at room temperature (23 to 25°C) on a standard medium, unless otherwise stated. The *mfr* mutation was isolated in a screen designed to recover EMS-induced male sterile mutations (Yamamoto, unpublished). The Oregon-R stock was used as the wild type control. A third chromosome multiple marker strain, *ru h th st cu sr e ca/TM3, Sb Ser*, and 13 deficiency strains were used to map the mutation. These deficiencies were as follows: *Df(3L)ZN47* (64C; 65C), *Df(3L)XD19* (65A2; 65E1) *Df(3L)RM5-2* (65E5; 66A17), *Df(3L)pbl-x1* (65F3; 66B10), *Df(3L)66C9-G28* (65B8-9; 66C9-10), *Df(3L)h-i22* (66D10; 66E1-2), *Df(3L)Scf-R11* (66E3-4; 66F1-2), *Df(3L)29A6* (66F5; 67B1), *Df(3L)AC1* (66A2; 67D13), *Df(3L)ldx6* (67E1-2; 68C1-2), *Df(3L)win5* (68A2; 69A1), *Df(3L)win6* (68C8-11; 69A4-5) and *Df(3L)cygC1* (69A4-5; 69D4-6). The *snky e/TM6B, e Tb* strain and strains of *ms(3)K81* and *gyn-F9* (*w; gyn-2; gyn-3*) were the gift of B. T. Wakimoto and Y. Fuyama, respectively. The other stocks used here were provided by the Bloomington stock center. Descriptions of genes and chromosome rearrangements are referred to Linsley and Zimm (1992) or FlyBase (http://flybase.bio.indiana.edu/).

Hatch rates. Fifty males of different genotypes (3–5 days old) and 40 Oregon-R females (3–5 days old) were placed in a culture vial for two days prior to egg collections. The flies were transferred to petri dishes containing fresh egg-laying media (25% apple juice, 1.25% sucrose and 1.75% agar) with yeast pastes. Eggs containing fresh egg-laying media (25% apple juice, 1.25% sucrose and 1.75% agar) with yeast pastes. Eggs obtained from overnight collection were counted twice and then allowed them to develop for at least 24 hrs. Unhatched eggs were counted twice to determine hatch rates. At least three separate trials were performed for each combination of *mfr, Df(3L)h-i22* and *snky*. *mfr/TM3* males were used as a control each time an experiment was done.

Egg collection and fixation. For initial characterization of mutant phenotypes, eggs laid by Oregon-R females mated with males of each genotype were collected within 30 min after egg deposition and were stored at 4°C to pre-
vent embryos from further development for up to 2 hrs before fixation. Eggs were washed in 1x PBS (1.9 mM NaH₂PO₄, 8.4 mM Na₂HPO₄, 175 mM NaCl, pH 7.4) containing 0.05% Tween-20 (PBST), and dechorionated in 50% commercial bleach for 2 min. For antibody staining to observe inseminating sperm, formaldehyde fixation was applied. The dechorionated eggs were washed and transferred into a two-phase fixative of heptane/PBS containing 3.7% formaldehyde and shaken for 20 min. The vitelline membranes were removed by transfer into a methanol/heptane mixture and vigorous shake until the most of eggs sank to the bottom of the methanol layer. Eggs were washed three times with methanol and stored in methanol at 4°C until use.

To compare the fertilization processes between wild type and mutant males, eggs were collected either within 15 min after egg deposition or directly from uterus. Because formaldehyde fixation of eggs during these very early stages results in high background fluorescence, dechorionated eggs were transferred into a methanol/heptane mixture and vigorously shaken until the most of eggs sank to the bottom of the methanol layer. Eggs were washed three times with methanol and stored in methanol at 4°C until use.

Staining. To determine if mfr sperm are competent to enter the egg, the eggs were stained with a mouse monoclonal antibody, DROP 1.1, that was reported to recognize the sperm tail in inseminated eggs (Karr, 1991; Graner et al., 1994), and was kindly provided by T. Karr. Fixed eggs as described above were incubated in hybridoma supernatants containing the primary antibody for 1 hr, washed in PBST for 1 hr, and subsequently stained with FITC-conjugated goat anti-mouse IgG (Jackson Labs) for 30 min. The eggs were counter-stained with 1 µg/ml PI for 30 min. After washing, the eggs were stained with DAPI for 5 min.

The stained eggs were mounted under the coverslips with 90% glycerol in PBS. All the staining and washing was performed at room temperature. Eggs were observed using either epifluorescent optics on NIKON ECLIPSE E600 microscope with filters for FITC (BA 515–555 nm/EX 465–495 nm), PI (GA; BA 590 nm/EX 510–560 nm) and DAPI (BA 435–485 nm/EX 340–380 nm), or OLYMPUS FLUOVIEW FV500 confocal laser microscope.

RESULTS

Isolation of mfr. As a part of the project designed to identify genes involved in male reproduction, we screened for male sterile mutations from natural populations, EMS-treated, and P element insertion lines. A total of 84 mutations were obtained (Yamamoto, unpublished). The mutations were classified into several types based on their phenotypes. Four recessive male sterile mutations, whose males produce large quantities of motile sperm that are transferred to female upon copulation, and stored in her sperm storage organs, but fail to produce progeny, were obtained. These mutations are the best candidates for paternal effect mutations. One of the mutations, designated misfire (mfr), was recovered from a collection of EMS-mutagenized third chromosome male sterile mutant lines.

Mapping of mfr. The mfr mutation was first mapped by recombination with respect to chromosomal markers, ru h th st cu sr e ca. Females heterozygous for a chromosome carrying mfr and a multiple marker chromosome were mated with mfr/TM3, Sb Ser males, and the Sb’ Ser+ sons then individually mated with two females carrying a multiple marker chromosome to check his fertility and chromosome composition. Of 106 males with recombinant chromosomes, none of them with h were fertile, indicating that the mutation was very close to the h locus. Calculating with ru and th markers, mfr was mapped to 3-28. The mutation was then mapped by complementation tests with 13 deficiency chromosomes deleted around the h locus. One of the deficiency chromosomes, Df(3L)h-i22, failed to complement mfr. Therefore, mfr was mapped to the cytological interval 66D10 to 66E1-2.

Analyses on fertility. To quantify the effects of the mfr on male fertility, we measured hatch rates of eggs laid by wild type females mated with mutant males (Table 1). While heterozygous mfr/TM3 males had normal levels of fertility, both homozygous mfr and heterozygous mfr/Df(3L)h-i22 males showed almost no fertility, with hatch rates of less than 0.1%, indicating that mfr mutation is likely a complete loss-of-function mutation. The very few escapers survived to adulthood, and appeared morphologically normal.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. eggs</th>
<th>No. embryos hatched</th>
<th>Hatch rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mfr/TM3</td>
<td>5465</td>
<td>4636</td>
<td>84.83</td>
</tr>
<tr>
<td>mfr</td>
<td>9897</td>
<td>5</td>
<td>0.05</td>
</tr>
<tr>
<td>mfr/Df(3L)h-i22</td>
<td>4852</td>
<td>3</td>
<td>0.06</td>
</tr>
<tr>
<td>snky/TM6</td>
<td>2240</td>
<td>2089</td>
<td>93.26</td>
</tr>
<tr>
<td>snky</td>
<td>4882</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>mfr/snky</td>
<td>1908</td>
<td>1821</td>
<td>95.44</td>
</tr>
</tbody>
</table>
In order to examine if the mfr mutant can initiate pronuclear maturation or fusion with the female pronucleus after entering into the egg, mfr males (w+/Y) were mated with either w or w;gyn-F9 females which bear two gynogenesis mutations on the 2nd and 3rd chromosomes (gyn-2 and gyn-3, respectively). The gyn-F9 females produce progeny (mainly X/X daughters with rare sterile X/O sons) at a high frequency gynogenetically when crossed to males of a paternal effect mutation, ms(3)K81 (Table 2, and Fuyama, 1986b). It should be stressed here that ms(3)K81 sperm form male pronuclei which are capable to fuse to female pronuclei (Fuyama, 1984; Yasuda et al., 1995). The mfr locus is clearly different from that of snky (cytologically, Fitch and Wakimoto, 1998). The normal fertility of the males heterozygous for mfr and snky (Table 1) suggests that trans-heterozygous genetic interactions are not present between these two mutations.

**Sperm entry and initiation of mitosis.** To determine if mfr sperm effectively enter the eggs, we examined sperm entry rates 30 min after egg deposition using DROP 1.1, an antibody that recognizes sperm tails in the inseminated eggs (Karr, 1991; Graner et al., 1994) and fertilization rates using a nuclear dye, DAPI. In heterozygous mfr/TM3 males, 97.8% of the eggs were inseminated and 97.2% of them contained mitotic nuclei (Table 3), as expected for normal fertilization. In contrast, although more than 90% of the eggs had sperm in both homozygous mfr and heterozygous mfr/Df(3L)h-i22 males, almost all of them appeared to be arrested prior to the initiation of mitosis (Table 3). These observations indicate that sperm of mfr are competent to enter into the eggs but fail to initiate mitotic divisions. In the present study, only 61.9% of the eggs were detected to have a sperm of snky males (Table 3). Although this percentage is somewhat lower than that as described in Fitch and Wakimoto (1998), inseminated eggs also failed to initiate embryogenesis.

**Sperm tail morphology in the egg.** In most of *Drosophila* species, the entire sperm enters into the egg (Karr and Pitnick, 1996). The highly stereotypical folding and coiling of the sperm tails are observed in the anterior end of the egg and the tail undergoes changes in morphology and position during embryogenesis (Karr, 1991, 1996; Pitnick and Karr, 1998), which raise an intriguing possibility that the sperm tail structure has essential roles in fertilization and/or following embryogenesis (Karr, 1991, 1996). For instance, in *D. melanogaster*, a significant portion of the tail is seen to coil around the circumference of the egg perpendicular to the A/P axis at fertilization, and this structure remains during the early stages of mitotic divisions (Fig. 1, A and B). Then, the entire tail is mildly folded in the anterior end of the egg (Fig. 1, C and D) and, by the time of syncytial blastoderm, the tail becomes tightly folded and moves away from the anterior end into the yolk region.

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### Table 2. Genetic examination of gynogenetic reproduction with the paternal effect sterile mutations

<table>
<thead>
<tr>
<th>Female genotype</th>
<th>Male genotype</th>
<th>No. males</th>
<th>Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>male</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[w]</td>
</tr>
<tr>
<td>w; gyn-2; gyn-3</td>
<td>+</td>
<td>15</td>
<td>444</td>
</tr>
<tr>
<td>w; gyn-2; gyn-3</td>
<td>ms(3)K81</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>w; gyn-2; gyn-3</td>
<td>snky</td>
<td>30</td>
<td>1b</td>
</tr>
<tr>
<td>w; gyn-2; gyn-3</td>
<td>mfr</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>w; +; +</td>
<td>mfr</td>
<td>30</td>
<td>5</td>
</tr>
</tbody>
</table>

*Sterile sons (most probably XO males) originated from gynogenetic reproduction.
Fertile sons (XY males) originated from normal reproduction.

### Table 3. Frequencies of sperm entry and fertilization

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. eggs</th>
<th>No. eggs with sperm</th>
<th>Sperm entry (%)</th>
<th>No. embryos fertilized</th>
<th>Fertilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mfr/TM3</td>
<td>184</td>
<td>180</td>
<td>97.8</td>
<td>179</td>
<td>97.2</td>
</tr>
<tr>
<td>mfr</td>
<td>259</td>
<td>241</td>
<td>93.1</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>mfr/Df(3L)h-i22</td>
<td>174</td>
<td>163</td>
<td>93.7</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>snky</td>
<td>160</td>
<td>99</td>
<td>61.9</td>
<td>1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Embryos initiated mitotic divisions were counted*
Fertilization defect in \textit{mfr} sperm

Although components of the sperm are clearly lost during these stages, the sperm tail persists throughout embryonic development and is eventually eliminated during larval development (Pitnick and Karr, 1998).

The shapes and positions of \textit{mfr} sperm tails were assorted into seven classes (Fig. 2). Classes 1 to 3 are commonly observed in the normal development (Fig. 2, A to C; see also Fig. 1). In order to compare the tail morphology in the same genetic background, we used eggs inseminated by heterozygous \textit{mfr}/TM3 males as the control. The eggs containing \textit{mfr}/TM3 sperm essentially belong to the three classes as illustrated in Fig. 3. However, approximately 45% of eggs inseminated by homozygous \textit{mfr} males contained sperm tail structures that deviated considerably from the three classes. These structures were rarely observed in \textit{mfr}/TM3 males (Fig. 3). For instance, a significant number of eggs had sperm that were not localized to the anterior region and were either relatively loosely coiled (Class 4, Fig. 2D) or simply coiled in a distinctly different topology (Class 5, Fig. 2E). Pronuclear fusion invariably occurs at approxi-
Fertilization defect in \textit{mfr} sperm

mately 75\% E.L. (= egg length, the anterior pole is 100\% E.L.) and the sperm tails generally do not pass through 50\% E.L. However, \textit{mfr} sperm sometime reached to posterior part of the egg (Class 6, Fig. 2F) and most part of these sperm was usually located on the periphery of the egg, indicating that these sperm failed to access the inner yolk region of the egg. In addition, a large part of sperm was occasionally observed to extend from the egg (Class 7, Fig. 2G). These eggs were unlikely to be fixed during sperm entry. If the eggs were fixed during sperm entry, the outside part of sperm is expected to be the posterior. The outside parts of the tail were invariably either the anterior part, including sperm head, and/or the middle part, and the posterior part of the tail was usually located in the egg cytoplasm. The last two classes indicate that \textit{mfr} sperm have a lowered capacity to penetrate the egg cytoplasm. Virtually all eggs inseminated by \textit{mfr} sperm arrested prior to the first mitotic division and 55\% of the eggs contained sperm tail structures essentially identical to wild type, suggesting that neither abnormal structures nor lowered capacity of penetration is the principal cause of sterility. Sperm of heterozygous \textit{mfr}/\textit{Df(3L)h-i22} males and homozygous \textit{snky} males also showed similar distributive patterns to those of \textit{mfr} males (Fig. 3).

\textbf{Sperm head morphology in the egg.} Staining \textit{eggs} inseminated by \textit{mfr} sperm with the nuclear dye DAPI revealed normally appearing four female meiotic products, one was the female pronucleus and the other three were polar bodies. The polar bodies were located at the surface of the egg and often aggregated to form a single group of chromosomes (Fig. 4, A and B) similarly in the normal development (Foe et al., 1993; Lassy and Karr, 1996). A single, needle-shaped fluorescent sperm head was also observed to be located at the surface of the egg (Fig. 4C). The merged view of DRO1.1 and DAPI staining images clearly indicated that this needle-shape structure is sperm nucleus (Fig. 5). Closer examination always identified the sperm head in the yolk-free cortical layer (Fig. 5, A and E). The localization of \textit{mfr} sperm was obviously different from that of \textit{mfr}/\textit{TM3} sperm which is in the yolk (see Fig. 4G). This result indicates that the heads of inseminating \textit{mfr} sperm are remained at the periphery of the eggs.

To compare the early stages of fertilization processes among \textit{mfr}, \textit{mfr}/\textit{TM3} and wild type (Oregon-R), eggs collected within 15 min after egg deposition, or directly from uterus, were fixed and nuclei were observed using DAPI (Table 4). In contrast to eggs inseminated by \textit{mfr} sperm, eggs laid by females mated with \textit{mfr}/\textit{TM3} or wild type males showed needle-shaped sperm heads at a low frequency (1.4\%). However, the vast majority (90\%) displaying typical nuclear bodies indicative of completion of karyogamy and initiation of the development of diploid embryos. Eggs collected form uterus also showed similar distributive patterns (data not shown). In contrast, 98\% of inseminating \textit{mfr} sperm retained needle-shape morphology and at most 2\% of them appeared normal (likely to be exceptional escapers that would survive to adult flies). This pattern was also applicable to \textit{snky}. These results strongly suggest that the primary lesion of \textit{mfr} sperm is a failure in proper initiation of the nuclear decondensation.

\textbf{Plasma membrane breakdown covering the head region of sperm.} Fluorescent nuclear dyes differ in their ability to penetrate through membranes. Fitch and Wakimoto (1998) reported that inseminating \textit{snky} sperm could be stained with the membrane-permeant nuclear dye DAPI but failed to stain with the membrane-impermeant nuclear dye PI. They suggested that this phenomenon is due to defect in breakdown of membranes (plasma membrane and nuclear envelope) surrounding the nuclear region of inseminating \textit{snky} sperm. To see if this phenomenon could apply to inseminating \textit{mfr} sperm, we doubly stained eggs with DAPI and PI. In \textit{mfr}/\textit{TM3} sperm, PI staining showed fluorescence as strong as DAPI staining (Fig. 4G and 4H). In contrast to the result of Fitch and Wakimoto (1998), \textit{snky} sperm stained weakly with PI (Fig. 4F), most probably due to differences in fixation. However, this staining was far weak when compared to DAPI staining of the sperm head (Fig. 4E) or PI staining of the polar body (Fig. 4B). \textit{mfr} sperm showed similar staining patterns (Fig. 4C and 4D), suggesting that \textit{mfr} sperm are also defective in breakdown of membranes surrounding the nuclear region of inseminating sperm.

The antigen recognized by DRO1.1 is suggested to be a class of heparan sulfate proteoglycans (Graner et al., 1994), which are often linked to the cell surface directly.

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\textbf{Fig. 2.} Variations in shapes and positions of sperm tails in eggs. Eggs inseminated by sperm of homozygous \textit{mfr} or heterozygous \textit{mfr}/\textit{Df(3L)h-i22} males are stained with DRO1.1 to visualize sperm tails. The sperm tail morphologies can be grouped into seven classes. (A) Class 1: a significant portion of the tail is seen to coil around the circumference of the egg perpendicular to the A/P axis; usually observed at fertilization and during early stages of embryogenesis (see Fig. 1A) in normal development. (B) Class 2: the entire tail is mildly folded and associated with the anterior end of the egg. (C) Class 3: the tail is tightly folded and positioned away from the anterior end of the egg. These latter two classes are also observed in normal development (see Fig. 1, B and C). Note, however, that these eggs are arrested prior to fertilization. The following classes were rarely observed during normal embryogenesis. (D) Class 4: the tail is unfolded and away from the anterior end of the egg. (E) Class 5: a significant portion of the tail is coiled but the coil is not associated with the anterior end of the egg. (F) Class 6: the tail extends into the posterior part of the egg. (G) Class 7: a significant portion of the tail remains outside the egg surface. Anterior is to the left. Bar represents 100 µm.
(syndecans) or through the anchor protein (glypicans; Bernfield et al., 1999). DROP 1.1 has been believed to stain sperm tails not sperm heads of D. melanogaster (Karr, 1991). However our examination at a higher magnification clearly confirmed that DROP 1.1 recognized not only sperm tails but also the head region (Fig. 5). If DROP 1.1 does stain the plasma membrane, the staining would disappear when sperm plasma membrane was degraded in the process of fertilization. As expected, DROP 1.1 staining around the nuclear region of sperm head was missing just after the beginning of nuclear decondensation (Fig. 6). It is interesting to note that the apical end of the sperm head, where the acrosome is present, was stained with DROP 1.1 (arrowhead in Fig. 6). Similarly, mfr sperm head, including the apical tip, was strongly stained with DROP 1.1 (arrowheads in Fig. 5). At a low frequency (<10%), a small inner portion around the distal end of the nuclear region was stained apparently less weakly as compared with the other region (Figs. 5F to 5H). We also carried out the analysis of snky sperm and found the same staining characteristics in the head region including the condensed nuclei as seen in mfr (data not shown).

**DISCUSSION**

**The defects of the mfr mutation.** In this paper, we describe the phenotype of the male sterile mutation mfr. Mutant males produce morphologically normal sperm, which are transferred during copulation and stored in the female sperm storage organs. Most eggs laid by females mated with mfr males were inseminated, but rarely showed any evidence of mitotic divisions suggesting that mfr+ gene plays an essential role in fertilization. During normal fertilization, the sperm nucleus undergoes a series of steps to form a male pronucleus, including the removal of the sperm nuclear envelope, decondensation of sperm chromatin and replacement of sperm chromosomal proteins with maternally provided histones, assembly of a nuclear envelope, lamina and matrix, and a final step of nuclear swelling before entry into the first embryonic S phase (Wright, 1999). The majority of eggs inseminated by mfr sperm contained a condensed, needle-shaped sperm nucleus, indicating that mfr sperm failed to undergo chromatin decondensation. The mutation is recessive and most probably represents a complete loss-of-function mutation, since homozygous mfr males showed the same defective phenotype as those seen in males heterozygous for a deficiency of the locus. The abnormality is restricted to male fertility, thus mfr is likely to be a strict paternal effect mutation.

Many aspects of Drosophila fertilization are similar to the other animals, but there are a few unique features (Karr, 1996). In many organisms, the fusion of sperm and egg plasma membrane, generally referred to as “syn-
Fig. 4. Staining of eggs inseminated by sperm of mfr (A to D), snky (E, F) and mfr/TM3 (G, H) with DAPI (A, C, E, G) and PI (B, D, F, H). Images A and B are polar body nuclei, and those from C to H are sperm heads. Arrowheads indicate sperm nuclei. Note that mfr sperm heads are stained with PI (D) very weakly as compared with DAPI (C), but mfr/TM3 sperm is stained with PI (H) as extensively as DAPI (G). Bar represents 10 µm.
Fig. 5. The head morphologies of mfr sperm in inseminated eggs. Sperm stained with DAPI (B, F), DROP 1.1 (C, G), and merged images (D, H). Images B, C, D and F, G, H are enlargements of the sperm head region marked in A and E, respectively. Arrowheads indicate the acrosome region; arrows indicate weakly stained region by DROP 1.1. The weakly stained gap region (G, H) is found at a low frequency in the mfr sperm. Bars represent 10 µm.
fertilization defect in *mfr* sperm

## Table 4. Developmental stages of eggs collected in 15 min of oviposition

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. eggs</th>
<th>Developmental stage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>847</td>
<td>1.4</td>
</tr>
<tr>
<td>mfr/TM3</td>
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<tr>
<td>mfr</td>
<td>264</td>
<td>98.1</td>
</tr>
<tr>
<td>snky</td>
<td>244</td>
<td>99.2</td>
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</table>

NS, needle-shaped (condensed) sperm nuclei; DS, decondensed sperm nuclei or male pronuclei; EC, early cleavage divisions (cycles 1–9); BL, blastoderm; GA, gastrulation or later.

Gamy”, occurs immediately after sperm-egg binding. Sperm penetration in *D. melanogaster* does not involve membrane fusion and the sperm enters apparently by puncturing a hole in the egg oolemma (Perotti, 1975). As a result, sperm enters into the egg with its plasma membrane intact. Therefore, the first step in the male pronuclear formation should be the removal of the sperm plasma membrane, at least, in the head region. Our data suggests that *mfr* sperm are defective in the plasma membrane breakdown. Differential staining of the sperm nucleus with membrane-permeant and -impermeant nuclear dyes showed that the nucleus of inseminating *mfr* sperm was intensely stained with membrane-permeant dye DAPI (Fig. 4C), but was only weakly stained with the membrane-impermeant dye PI (Fig. 4D) suggesting the presence of an intact plasma membrane. However, this data did not allow us to distinguish whether both plasma membrane and nuclear envelope were affected by *mfr* or only either of them. Another, more direct evidence came from the observation that the entire head of inseminating *mfr* sperm was recognized by an antibody, DROP 1.1 (Fig. 5) which has been reported to recognize a family of heparan sulfate proteoglycans (Graner et al., 1994). This result indicates that plasma membrane covering the head region of the inseminating *mfr* sperm remained intact or affected processes related to membrane breakdown.

*mfr* sperm were also abnormally localized in the inseminated egg. This was easily observed by visualizing heads and tails of the inseminating sperm. Since the entire sperm that is 1.9 mm in length enters egg cytoplasm (Karr, 1991), and the highly stereotypical folding and coiling of the tail whose morphology and position changes during embryogenesis are observed, it has been suggested that the sperm tail and/or its structural change have important roles in embryogenesis (Karr, 1991, 1996). As pointed out by Karr (1991, 1996) because the head remains physically attached to the sperm tail, its position in the egg is determined by the overall coiling and folding of the entire structure. Given that over 90% of *mfr* sperm heads in inseminated eggs are found in the egg periphery and not in their normal location deep within the egg cytoplasm, it is reasonable to assume that the *mfr* mutation affects the overall folding and coiling of the sperm. This is certainly true for that portion of the sperm immediately proximal to the sperm head. However, because about 55% of inseminating *mfr* sperm showed typical folding and coiling patterns as seen in the normal developments (classes 1 to 3 in Fig. 3), sperm tail coiling does not explain all of the sterility caused by *mfr*. Therefore, while clearly *mfr* sperm do not properly enter the egg upon fertilization, this data cannot distinguish between cause and effect and could merely reflect the downstream consequences of an earlier defect in sperm-egg interactions. As discussed below, one such primary lesion in *mfr* sperm may involve breakdown and removal of the plasma membrane.

### Removal of plasma membrane covering sperm head during fertilization.

Although mechanisms of plasma membrane breakdown during *Drosophila* fertilization remain unclear, it is most likely that the interaction of sperm plasma membrane with egg cytoplasmic factors promotes the breakdown. Removal of plasma membrane covering the head region of inseminating sperm allows exposure of the sperm nucleus to the egg cytoplasmic factors, which leads the sperm nucleus to following steps for the male pronuclear formation. As mentioned previously, the *mfr* mutation is nearly a complete loss-of-function mutation. Expressivity of the *mfr* mutant phenotype was high, but not complete. A small percentage of the eggs developed into the adult flies that were not produced by parthenogenesis (Table 2), which was also observed in the *snky* mutation (Table 2; Fitch and Wakimoto, 1998). As shown in Figs. 5E to H, in some inseminating sperm, a part of plasma membrane seemed to be degraded at a low frequency.

Although a part of plasma membrane seemed to be degraded in *mfr* inseminating sperm, the most apical end of the sperm head region was likely to retain plasma membrane (Fig. 5E to 5H). Like other animals, the apical region of *Drosophila* sperm contains the acrosome (Perotti, 1969). In mammals, shortly after binding to the egg zona pellucida (ZP), sperm undergo cellular exocytosis, called the acrosome reaction. Only the sperm that have completed the acrosome reaction can penetrate the ZP and fuse with the egg plasma membrane (Abou-Haila and Tulsiani, 2000; Flesch and Gadella, 2000). The
The acrosome reaction involves multiple fusions between outer acrosomal membrane that underlies the plasma membrane and plasma membrane at the apical region of the sperm head, and exposure of acrosomal contents (Abou-Haila and Tulsiani, 2000; Flesch and Gadella, 2000). Therefore, plasma membrane covering the acrosome is degenerated into small fragments after the acrosome reaction. Although nothing is known about roles of the acrosome in *Drosophila*, it is unlikely to have an analogous role to that known in mammals after sperm entry into egg, since the plasma membrane of the sperm found inside the egg is retained intact (Fig. 6). It could be speculated that the acrosome reaction might be required for removal of plasma membrane covering head region of inseminating sperm, and the *mfr* gene functions in some process of the acrosome reaction. Although nothing is known about roles of the acrosome in *Drosophila*, it is unlikely to have an analogous role to that known in mammals after sperm entry into egg, since the plasma membrane of the sperm found inside the egg is retained intact (Fig. 6). It could be speculated that the acrosome reaction might be required for removal of plasma membrane covering head region of inseminating sperm, and the *mfr* gene functions in some process of the acrosome reaction. However, in the normal fertilization, the plasma membrane at the apical region remains intact even at the initiation of male pronuclear formation. This observation suggested that the acrosome reaction is unlikely to be the prerequisite for the multiple fusion of membranes in the process of plasma membrane breakdown surrounding the nuclear region. The acrosome function in *Drosophila* fertilization remains a mystery.

**Function of the *mfr* gene product.** Any model proposed for the *mfr* mutant phenotype must take into account two observations: inseminating *mfr* sperm retains its plasma membrane in the apical head region and the head is mislocalized to the periphery of the egg. Two alternative models could explain these phenotypes. First, the primary defect in *mfr* sperm could prevent removal of the plasma membrane surrounding the inseminating sperm head which could delay, or eliminate exposure of the basal body as well as the sperm nucleus to the egg cytoplasmic factors. The basal body carries a centriole pair that generates the microtubule organizing center used to construct the spindle poles for the first mitotic division (Foe et al., 1993; Callaini and Riparbelli, 1996). Similarly, the microtubule organizing center is essential for constructing the sperm aster that is indispensable for pronuclear migration in sea urchin (Schatten and Schat-
Fertilization defect in mfr sperm

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REFERENCES


