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Paternal Chromosome Incorporation into the Zygote Nucleus Is Controlled by *maternal haploid* in *Drosophila*

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maternal haploid **(*mh*) is a strict maternal effect mutation that causes the production of haploid gynogenetic embryos (eggs are fertilized but only maternal chromosomes participate in development). We conducted a cytological analysis of fertilization and early development in *mh* eggs to elucidate the mechanism of paternal chromosome elimination. In *mh* eggs, as in wild-type eggs, male and female pronuclei migrate and appose, the first mitotic spindle forms, and both parental sets of chromosomes congress on the metaphase plate. In contrast to control eggs, *mh* paternal sister chromatids fail to separate in anaphase of the first division. As a consequence the paternal chromatin stretches and forms a bridge in telophase. During the first three embryonic divisions, damaged paternal chromosomes are progressively eliminated from the spindles that organize around maternal chromosomes. A majority of *mh* embryos do not survive the deleterious presence of aneuploid nuclei and rapidly arrest their development. The rest of *mh* embryos develop as haploid gynogenetic embryos and die before hatching. The *mh* phenotype is highly reminiscent of the early developmental defects observed in eggs fertilized by *ms(3)K81* mutant males and in eggs produced in incompatible crosses of *Drosophila* harboring the endosymbiont bacteria *Wolbachia*.**

Key Words: maternal haploid; Drosophila; **fertilization; mutation; pronucleus formation; chromosome segregation.**

INTRODUCTION

Animal fertilization brings together two haploid gametes which are very different in aspect and must cooperate to form the zygote. The oocyte contains a large amount of cytoplasm able to support early embryonic development and a nucleus which is usually arrested in meiosis until egg activation and fertilization. In contrast, the cytoplasmic compartment of the sperm cell is reduced and is mainly featured by the sperm tail and the basal body. The sperm chromatin is packaged by sperm-specific chromosomal proteins, reaching an extremely condensed state during late stages of spermatogenesis. At fertilization, the sperm nucleus must be transformed into a DNA replication-competent and mitotically active male pronucleus in coordination with the female counterpart. The remodeling of the paternal chromatin involves a series of steps that appear conserved among species. They include, successively, the removal of the sperm nuclear envelope; the decondensation of the sperm chromatin and the replacement of sperm chromosomal proteins with maternally provided histones; the assembly of a nuclear envelope, lamina, and matrix; and a final step of nuclear swelling before entry into the first embryonic S phase (for review, see Poccia and Collas, 1996; Wright, 1999). Biochemical analyses of egg extracts from various model organisms such as *Xenopus*, sea urchin, surf clams, or *Drosophila* have led to isolation of several factors that allow sperm chromatin decondensation and pronuclear assembly *in vitro* (Poccia and Collas, 1996; Wright, 1999). Genetic analysis provides a complementary approach for the understanding of male pronucleus formation *in vivo*. In this regard, a few parental effect mutations affecting the participation of the male complement in the diploid zygote have been recently described in *Drosophila* (Yasuda *et al.*, 1995; Fitch and Wakimoto, 1998; Loppin *et al.*, 2000; for reviews see Foe *et al.*, 1993; Karr, 1996; Fitch *et al.*, 1998). These mutations induce three different phenotypic classes.

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First, several paternal effect mutations share a sperm activation defective phenotype characterized by the complete absence of sperm decondensation and zygote formation (Fitch *et al.*, 1998). For example, the sperm produced by *sneaky* (*snky*) mutant males is unable to lose its cell membrane after fertilization (Fitch and Wakimoto, 1998). Consequently, the sperm nucleus conserves its original needle shape; the sperm centrosome is not delivered to the oocyte cytoplasm, and the embryo development does not start. The second phenotypic class is featured by the maternal effect mutation *sésame* (*ssm*), which impedes a late stage of sperm chromatin decondensation (Loppin *et al.*, 2000). In this case, the condensed male pronucleus is excluded from the first mitotic spindle which contains only the maternal chromosomes and haploid development proceeds. Finally, the third phenotypic class is represented by the paternal effect mutation *ms(3)K81* (*K81*) (Fuyama, 1984; Yasuda *et al.*, 1995), which affects the division of paternal chromosomes during early development, suggesting a delayed influence of sperm chromatin remodeling on mitotic paternal chromosomes division. When crossed with wild-type females, homozygous *K81* males produce a majority of early dying embryos but a small fraction develops further as haploid gynogenetic embryos. *Drosophila* parental effect mutations associated with the production of haploid gynogenetic embryos such as *K81* and *ssm* potentially affect either the formation of the male pronucleus or the participation of the paternal complement to the formation of the zygote. The first mutation described with such a phenotype was *maternal haploid* (*mh*) (Santamaria and Gans, 1980). *mh*, originally named *fs(1)1182*, was isolated in a mutagenesis designed to recover recessive female sterile mutations on the X chromosome [*fs(1);* Gans *et al.*, 1975; Zalokar *et al.*, 1975]. Several other mutations producing haploid embryos from two other complementation groups were also obtained in this screen (Zalokar *et al.*, 1975) but only *mh* was conserved (P. Santamaria, personal communication). Haploid embryos produced by *mh* females have been used for studying haploid development (Santamaria and Gans, 1980; Santamaria, 1983; Edgar *et al.*, 1986; Sullivan, 1987) or for isolating haploid cell lines (Debec, 1978). However, the mechanism of paternal chromosome elimination in *mh* embryos remained unsolved. In this paper, we report a detailed cytological analysis of the *mh* phenotype to understand the origin of paternal chromosomes exclusion in early *mh* embryos. We observed, similar to Liu *et al.* (1997a), that both pronuclei form, migrate, and appose in *mh* eggs. However, the paternal chromatids are unable to separate in anaphase of the first embryonic mitosis and form a chromatin bridge. As a consequence, haploid nuclei of maternal origin rapidly separate from the damaged paternal chromosomes and haploid embryos develop. *sésame* and *maternal haploid* are the only reported maternal effect mutations which specifically prevent paternal chromosomes from participating in embryonic development. *mh* clearly belongs to the *K81* phenotypic class, suggesting that both maternal and paternal products control related steps of sperm chromatin transformation at fertilization.

MATERIALS AND METHODS

Drosophila Strains

All strains were raised at 25°C on standard media. The *y* w^a *v mh/FM7c* strain used in this study was generously provided by P. Santamaria. The EMS-induced *fs(1)1182* mutation was originally described as a recessive, thermosensitive female sterile X-linked mutation (Gans *et al.*, 1975; Zalokar *et al.*, 1975). *fs(1)1182* was renamed *maternal haploid* (*mh*) (Santamaria and Gans, 1980) and a nonthermosensitive strain of *mh* was isolated (Santamaria, 1983). *mh* was first mapped by recombination between the eye markers *vermillon* (*v*) (10A1-2) and *garnet* (*g*) (12B6-7; Debec, 1978; cytological positions on polytene chromosomes used in this paper are from Lindsley and Zimm, 1992). In agreement with this localization, a rapid recombination analysis of the *mh* chromosome allowed us to map the mutation between *vermillon* and *forked* (*f*; 15F1-3; data not shown). In addition, the following X chromosome deficiencies were used in complementation tests with *mh: Df(1)HA32; Df(1)ct268-42; Df(1)ct4b1; Df(1)v-N48; Df(1)m13; Df(1)N105; Df(1)JA26; Df(1)C246; Df(1)g; Df(1)RK2; Df(1)RK3; Df(1)RK4; Df(1)sd72b; Df(1)19; Df(1)r-D1;* and *Df(1)B25*. All these deletions complemented the strict female sterility phenotype of *mh*. Taken together, these results suggest that *mh* could fall within the small gap (12A1; 12A3-10) not covered by the currently available deficiencies of the *v–g* region. We obtained a very low hatching rate (~3%; $n = 611$) of embryos from *mh/Df(1)RK4* females crossed with wild-type males. Dead embryos presented a very similar cuticular phenotype than that of *mh* haploid embryos (Fig. 1C). However, DAPI staining of syncytial embryos from *mh/Df(1)RK4* females revealed their diploidy (not shown), confirming the complementation between *mh* and *Df(1)RK4*. Thus, another maternal effect embryonic lethal mutation distinct from *mh* must be present in the 12F5-6; 13A9-B1 region on the *mh* chromosome. The late embryonic lethality associated with this putative second mutation did not interfere with our analysis of early developmental defects of *mh* embryos.

The ssm^{185b} *mh* and the $KLP3A^{1611}$ *mh* double-mutant strains were obtained by recombination between the *cv* ssm^{185b} *f* (Loppin *et al.*, 2000) and *y* w^a *ct v mh* chromosomes and the *y* $KLP3A^{1611}$ *cv v f* (a gift from B. C. Williams) and *y* w^a *v mh* chromosomes, respectively. The genotypes of the two double-mutant strains were verified by complementation and phenotypic analyses (not shown). The *P[dj-gfp]* strain was kindly provided by A. Santel. The X-deficiency stocks used in this study were generously provided by the Umea and Bloomington *Drosophila* stock centers (genotypes and breakpoint positions are available on the World Wide Web at http://Flybase.bio.indiana.edu/).

Fertility Tests and Cuticle Preparations

Virgin females of different genotypes were aged for 4 days at 25°C in the presence of males and were then allowed to lay eggs on regular medium for 15–20 h. Embryos were counted twice and then let develop for at least 36 h at 25°C. Nonhatched embryos were counted twice to determine hatching rates. For cuticle preparation, embryos were aged and collected in the same way. After dechorionation and devitellinization (see next section), embryos were

mounted in Hoyer's medium (Van Der Meer, 1977) onto slides that were incubated overnight at 60°C before observation. Cuticles were observed and scanned using the transmission mode of the confocal microscope.

Cytological Analysis of Embryonic Phenotypes

Eggs or early embryos were collected 15 min after egg deposition (AED) and were stored at 4°C for up to 2 h before fixation. Egg collection and fixation were essentially done as described before (Loppin *et al.*, 2000) with the exception that the acetone step was eliminated from the fixation protocol. For immunostaining experiments, fixed eggs or embryos were washed 3 times (10 min each) in TBST, 0.15% Triton X-100 (TBST is a 50 mM Tris–HCl pH 8, 150 mM NaCl solution). Fixed eggs or embryos were then incubated with primary antibodies in TBST, 0.15% Triton overnight at 4°C, and washed 3 times (20 min each) in TBST, 0.3% Triton before incubation with secondary antibodies in TBST, 0.15% Triton overnight at 4°C or 3 h at room temperature. Eggs were rinsed again in TBST, 0.3% Triton and incubated for 1 h in a 2 mg/ml RNAse A solution at 37°C. After rinsing once in TBST, 0.3% Triton, eggs were incubated 30–60 min in 5 μg/ml propidium iodide (PI) or 1 μM YO-PRO-1 (Molecular Probes) at room temperature. Embryos were washed in TBST, 0.3% Triton for 20 min and mounted in the same solution. Coverslips were sealed with nail polish before examination.

Antibodies

The monoclonal antibody ADL 67, directed against *Drosophila* lamin Dm_0 (Smith and Fisher, 1989), was kindly provided by P. Fisher and used at a 1:1 dilution. The rabbit polyclonal anti-CID and the anti-CP190 antibodies (Rb188 antiserum) were kindly provided by S. Henikoff and W. G. F. Whitfield and were used at dilutions 1:1000 and 1:500, respectively. Rabbit polyclonal anti-phospho-histone H3 antibody (Upstate Biotechnology, 06-570) was used at a 1:500 dilution. A monoclonal anti-α-tubulin antibody (Amersham Pharmacia Biotech) was used at a 1:100 dilution. Cy3- and Cy5-conjugated goat anti-rabbit IgG antibodies (Amersham Pharmacia Biotech) and Rhodol green-conjugated goat anti-mouse IgG antibodies (Molecular Probes) were used as secondary antibodies at a 1:300 dilution.

Confocal Microscopy and Imaging

Optical sections were made using a confocal laser scanning microscope (LSM 510, Zeiss). PI fluorescence was monitored using the He–Ne laser 543-nm excitation line and a long-pass 585-nm filter. Rhodol green fluorescence was monitored using the argon laser 488-nm excitation line and a band pass 510–530 nm filter. Dichroic mirrors (488/543 and 543 nm) were used when PI and Rhodol green signals were monitored simultaneously. This was achieved by using two independent photomultipliers. A line-per-line simultaneous image acquisition procedure was made possible by an accoustico–optical filter controlling the switch between laser excitation lines. For triple staining with Rhodol green, Cy3, and Cy5, we used successive scans of the frame with excitation lines 488, 543, and 633 nm, respectively, and the emitted fluorescence was filtered with corresponding band-pass filters 510–550, 560–615, and long-pass filter 680 nm. Z-series of optical sections were obtained in some cases and were projected along the Z axis to obtain a general view of the specimen. Images were further processed using Photoshop 5.0.2 (Adobe). Background fluorescence was removed from the digital images for a better demonstrativeness of the data, making sure that specific signals were not altered.

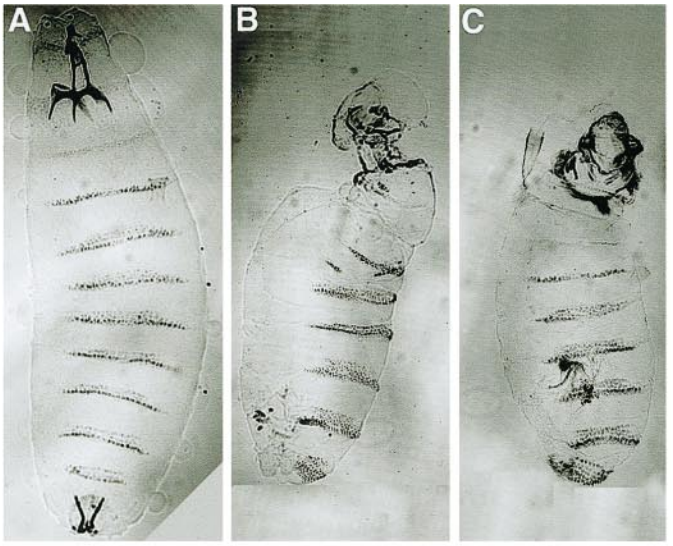
RESULTS

We first verified that embryos produced by homozygous *mh* females crossed with wild-type males (called *mh* embryos for clarity) never hatched ($n > 800$). We found that 22% ($n = 795$) of *mh* embryos reached late embryogenesis and deposited a cuticle before dying. Cuticles from *mh* embryos consistently displayed important head defects, antero–dorsal holes, and missing or abnormal segments (Figs. 1A and 1B; see also Edgar *et al.*, 1986). As previously reported (Zalokar *et al.*, 1975; Santamaria and Gans, 1980; Santamaria, 1983), we observed that these late-dying embryos were haploid gynogenetic embryos (not shown). We then investigated the phenotype of *mh* eggs to identify the mechanism of paternal chromosome elimination during early development.

Pronuclear Formation and Migration Occur Normally in mh Eggs

Although the gynogenetic haploid development of *mh* embryos was determined more than 20 years ago (Santamaria and Gans, 1980), the mechanism of paternal chromosome exclusion from the developing eggs remained unclear. Edgar *et al.* (1986) proposed a defect of pronuclear migration as the primary cause of haploid development. More recently, a report of Liu *et al.* (1997a) on pronuclear lamina formation in *Drosophila* presented a picture of apposed pronuclei in a fertilized *mh* egg. These contradictory data prompted us to reinvestigate the details of the cytology of fertilization and early development in *mh* eggs.

We crossed *mh* females with *P[dj-gfp]* males that produce sperm with a GFP-tagged tail (Santel *et al.*, 1997) and verified that most *mh* eggs contained a sperm tail (not shown). We also observed that female meiosis in *mh* eggs produced a female pronucleus and three polar bodies as in control eggs (Fig. 2D). In agreement with the observation of Liu *et al.* (1997a), we observed that pronuclei apposed in *mh* eggs (Fig. 2C). We stained eggs and early embryos with an antibody directed against histone H3 phosphorylated at serine 10 (PH3; Fig. 2). PH3 is present in newly replicated chromosomes from prophase to late anaphase and thus provides a useful marker for mitosis (Hendzel *et al.*, 1997; Wei *et al.*, 1998). As reported earlier, the PH3 staining on chromosomes of early *Drosophila* embryos is very intense in metaphase, weakens in early anaphase, and disappears at the poles of migrating chromatids in late anaphase (Su *et al.*, 1998). Typically, the telophase chromatin was not stained with the anti-PH3 antibody (Fig. 2G). In wild-type fertilized eggs, we observed that the female pronucleus was already in early prophase and stained with the anti-PH3 antibody, whereas the still interphasic male pronucleus contained no significant PH3 fluorescence (Fig. 2A). This



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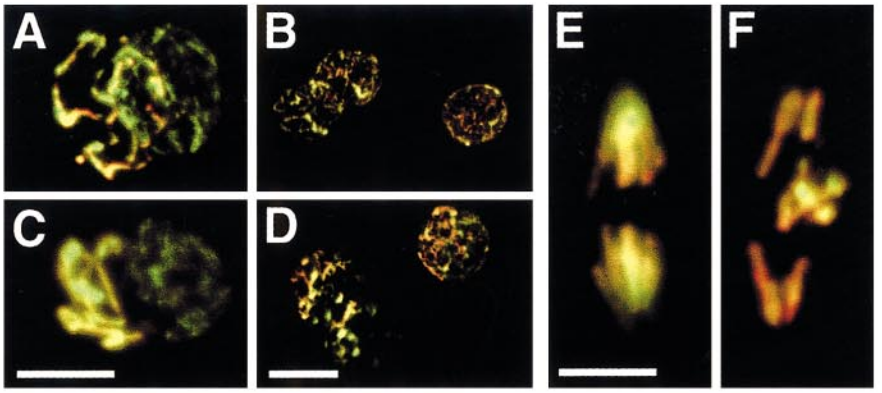

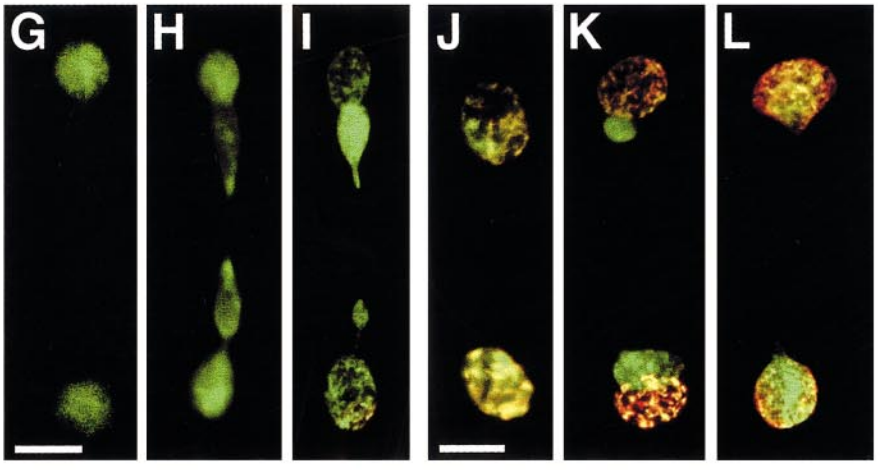

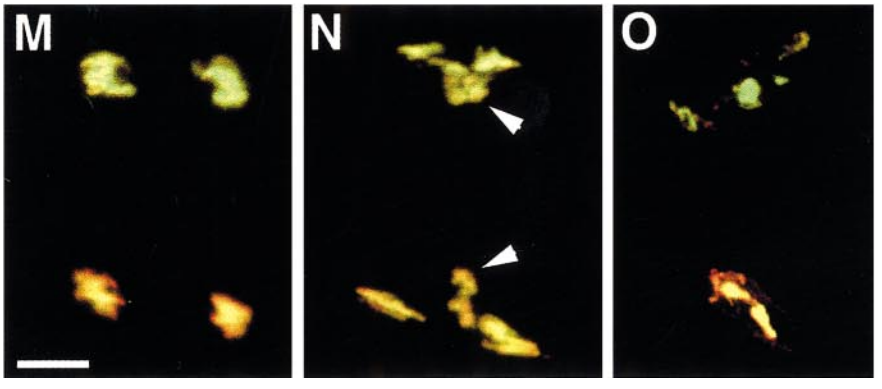

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confirmed previous observation of asynchronous entry of the two pronuclei in the first mitosis in *Drosophila* embryos (Sonnenblick, 1950; Callaini and Riparbelli, 1996). The same asynchrony between pronuclei was observed in *mh* fertilized eggs (Fig. 2C). We also observed a few *mh* eggs with nonapposed male and female pronuclei, but in similar proportion as found in control eggs (not shown). These cases probably represented eggs that were fixed during the migration of the female pronucleus from the antero–dorsal periphery of the egg toward its more centrally located male counterpart. Finally, at the pronuclear apposition stage, the three polar bodies produced at the second division of the female meiosis appeared identical in *mh* and control eggs (Figs. 2B and 2D). In conclusion, *mh* does not affect the formation and behavior of pronuclei until apposition.

The First Embryonic Mitosis Is Defective in mh Eggs

In *Drosophila*, pronuclear envelopes do not fuse and the two parental sets of chromosomes enter the first embryonic mitosis as separated entities, a process known as gonomery (Sonnenblick, 1950; Callaini and Riparbelli, 1996). From late prophase through late anaphase, each haploid set of chromosomes (or pairs of sister chromatids) occupies one-half of the gonomeric spindle. In telophase of the first division, the chromosomes decondense and the parental genomes mingle for the first time. We found that the gonomeric division in *mh* fertilized eggs was always defective (Table 1). Indeed, in anaphase of the first division, only one-half of the chromosomes migrated toward the spindle poles, whereas the other half was still lagging behind on the metaphase plate (Fig. 2F). In telophase, a chromatin bridge formed between the two daughter nuclei (Fig. 2H). At the end of the first division, the bridge broke down and the late chromatin segregated at random between the two zygotic nuclei (Figs. 2I and 2K). Considering the haploid gynogenetic development of *mh* embryos (Santamaria and Gans, 1980), we presumed that the set of lagging chromosomes in anaphase of the first mitosis in *mh* eggs contained only paternal chromosomes. This was indeed the case, as shown by analysis of *ssm mh* double-mutant eggs in the next section. In contrast to paternal chromosomes, maternally derived chromatids segregated normally before entering

TABLE 1
Mitotic Defects in the First Two Divisions of *mh* Embryos

Mitotic stage and phenotype	Wild-type			*mh*		
		n	%		*n*	%
Anaphase 1[a] half-late[b]	0	16	0	17	17	100
Telophase 1 with bridge	1	18	5	30	30	100
Interphase 2 with unfused complements	0	12	0	22	24	92
Mitosis 2: No defect	58	60	97	0	54	0
Mitosis 2: Half-late	0	—	0	26	—	48
Mitosis 2: Aneuploid	2	—	3	28	—	52

[a] The number corresponds to the nuclear cycle of the embryo.
[b] Half-late indicates the presence of lagging paternal chromosomes.

FIG. 1. Cuticular phenotype of *mh* embryos. Cuticles from a wild-type embryo (A), an *mh* embryo (B), and an embryo produced by a *mh/Df(1)RK4* female (C; see Materials and Methods). Note the same phenotype of embryos in (B) and (C) characterized by a disorganized head, an anterodorsal hole, missing abdominal segments, and an internalized telson. Cuticles are presented at the same scale with the anterior part up and the focus was made on the ventral side.

FIG. 2. *mh* affects the ability of paternal chromosomes to divide properly during the first embryonic mitosis. Wild-type or *mh* fertilized eggs or early embryos stained for DNA (green) and PH3 (red) were observed using laser scanning confocal microscopy. Chromatin containing phosphorylated histone H3 appears yellow. (A) Apposed pronuclei in a wild-type egg, with the female pronucleus (left) containing condensing preprophasic chromosomes stained for PH3. The decondensed male pronucleus has not yet entered the first mitosis and shows up in green (right). (B) The three interphasic polar bodies from the same egg at the periphery of the oocyte cytoplasm. (C, D) Pronuclei (C) and companion polar bodies (D) in a fertilized *mh* egg. Pronuclei are apposed and enter the first division asynchronously as control pronuclei. (E) Anaphase of the first mitosis in a wild-type embryo. (F) The first mitosis in an *mh* egg showing the female chromosomes in anaphase and the paternal chromosomes lagging on the metaphase plate. Note that the PH3 staining is more intense on the paternal chromosomes, suggesting that they are blocked in a metaphasic state. (G, H, I) Late telophase of the first division in wild-type (G) or *mh* eggs (H, I). In the control wild-type egg, both daughter nuclei contain a diploid content of decondensed chromatin (G), while in *mh* eggs, a chromatin bridge of paternal origin is formed between the two telophasic daughter nuclei (H). This situation leads to a random segregation of the paternal complement (I). As a consequence, a high incidence of aneuploidy is observed in *mh* embryos, in interphase of nuclear cycle 2 (K). The nuclear material which was contained in the bridge of the precedent division is not fused with the rest of the chromatin and does not stain yet for PH3. (L) A rare case of *mh* cycle 2 embryo containing fused sister nuclei (compare with the control embryo in J). (N) During the second division in a fraction of *mh* embryos, the lagging phenotype of paternal chromosomes (arrowheads) observed at the first mitosis is reproduced. More frequently, cycle 2 *mh* embryos display a marked aneuploidy with fragmented chromosomes (O). (M) shows a wild-type embryo in anaphase of nuclear cycle 2. Bars: 10 μm.

interphase of nuclear cycle 2 (Figs. 2H, 2I, 2K, and 2L). At this stage, maternal nuclei appeared most often physically separated from the late paternal chromatin and entered the next cycle well ahead as judged by the reappearance of the PH3 fluorescence (Fig. 2K). Daughter nuclei of apparently wild-type aspect were formed on rare occasions at the end of the first mitosis (Fig. 2L and Table 1).

In the second embryonic mitosis in *mh* embryos, we found two major phenotypic classes. About half of the embryos displayed the phenotype described for the first division with about half the chromosomes lagging behind (arrowheads in Fig. 2N, compare with Fig. 2F). Other cycle 2 *mh* embryos appeared aneuploid as they contained disorganized nuclei and fragmented chromosomes (Fig. 2O). The presence of lagging paternal chromatin in each zygotic nucleus was occasionally observed in the third nuclear cycle, but not in older *mh* embryos (see also Fig. 7).

mh Affects only Paternal Chromosomes

We presumed in the previous section that the abnormally dividing chromosomes in early *mh* embryos were of paternal origin. To verify that this was actually the case, we constructed double-mutant females bearing *mh* and the recently described maternal effect mutation *sésame* (Loppin *et al.*, 2000). The male pronucleus in fertilized eggs produced by homozygous *ssm* females is a small, abnormally condensed nucleus that is excluded from the first mitotic spindle. Consequently, *ssm* embryos are haploid and develop only with the maternally derived chromosomes (Loppin *et al.*, 2000). As expected, fertilized eggs from double-mutant *ssm mh* females contained a round, condensed male pronucleus indistinguishable from the male pronucleus in *ssm* eggs (Fig. 3). The elimination of the paternal complement from the first nuclear division allowed us to specifically observe the behavior of the maternal chromosomes during the first division in the *mh* mutant context. In anaphase of the first mitosis, the four pairs of maternal sister chromatids separated (Fig. 3) and bridges in telophase were not observed more frequently than in control embryos (not shown). This result shows a clear epistasis of *ssm* over *mh* with respect to male pronucleus formation. Furthermore, it demonstrates that *mh* does not interfere with the normal division of maternal chromosomes. We concluded that *mh* affects only paternal chromosomes.

Organization of the First Spindle in mh Fertilized Eggs

We used antibodies specific for tubulin and for the centrosomal protein CP19O (Whitfield *et al.*, 1988) to analyze the formation of the gonomeric spindle in *mh* eggs. In *Drosophila*, the fertilizing sperm brings into the egg the basal body from which the first centrosome of the embryo is presumably formed (Riparbelli *et al.*, 1997). After its duplication, the centrosome is first required for the formation of the sperm aster and later for the organization of the gonomeric spindle. The gonomeric spindle in *mh* embryos had a centrosome at each pole and seemed to adapt well to the asynchrony of both sets of parental chromosomes during mitosis (Figs. 4A and 4B). To test the ability of paternal chromosomes in *mh* eggs to form a mitotic spindle independently of the presence of maternal chromosomes, we constructed $KLP3A^{1611}$ *mh* double-mutant females. $KLP3A^{1611}$ is a maternal effect mutation which impedes the migration of the female pronucleus in fertilized eggs (Williams *et al.*, 1997). In fertilized $KLP3A^{1611}$ eggs, the paternal chromosomes are blocked in metaphase of the first division and the embryo does not develop. However, a mitotic spindle of normal aspect is organized around the isolated paternal complement (Williams *et al.*, 1997). We observed the same situation in eggs from $KLP3A^{1611}$ *mh* females: a spindle with centrosomes at both poles containing the haploid set of paternal chromosomes (Fig. 4C). Thus, the segregation defects of the paternal chromosomes in *mh* eggs are not a consequence of abnormal mitotic apparatus organization.

Centromeric Heterochromatin Distribution Is not Affected by the mh Mutation

To better understand the structure of paternal chromosomes in *mh* embryos and the nature of their association with the mitotic spindle apparatus, we stained *mh* fertilized eggs with an antibody directed against the centromeric histone H3-like protein Cid (Henikoff *et al.*, 2000). Cid is present in the pericentromeric region of *Drosophila* chromosomes throughout the cell cycle. At metaphase of the first division in *mh* eggs, both haploid sets of chromosomes contained a number of Cid spots of similar size and brightness (Fig. 5A). In anaphase, four spots could be distinguished at the tip of each bundle of migrating maternal sister chromatids. Lagging paternal chromosomes also had their centromeres oriented poleward but the corresponding chromatids did not fully separate (Fig. 5B). In telophase of the first division, maternal centromeres occupied the extremity of the nuclei, whereas paternal centromeres were distributed throughout the length of the chromatin bridge (Fig. 5C). The uneven segregation of paternal chromosomes at the first division was confirmed by the observation of preparations of nuclei from cycle 2 *mh* embryos where centromeres could be unambiguously numbered (Fig. 5D). We concluded that paternal chromosomes are able to form functional centromeres in *mh* eggs. Thus, the defect of paternal chromatids segregation is apparently not a consequence of an aberrant centromere formation or function.

How Are Haploid mh Embryos Generated?

We postulated that the development of haploid *mh* embryos requires the separation of at least one haploid maternal zygotic nucleus from the damaged paternal chromatin

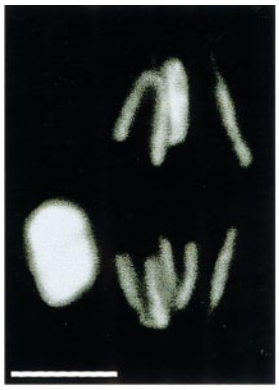
early in development. This was already suggested by the results presented in Fig. 2 where maternal daughter nuclei often appeared to enter nuclear cycle 2 independently of the late paternal chromatin. In order to strengthen this observation we investigated the reformation of nuclear lamina at the end of the first mitosis in *mh* embryos. The nuclear lamina is a filamentous protein meshwork lining the inner nuclear envelope in eukaryotes (for review, see Nigg, 1992; Georgatos *et al.*, 1994; Liu *et al.*, 1997b). In *Drosophila* embryonic syncytial divisions, the nuclear lamina breaks down in metaphase and begins to reform as soon as the next telophase (Paddy *et al.*, 1996). We stained early *mh* embryos with an antibody directed against *Drosophila* lamin Dm_0, a major component of the nuclear lamina (Smith and Fisher, 1989). We observed that the lagging paternal chromatin in telophase of the first division was surrounded by a nuclear lamina that was clearly distinct from the lamina present around the maternally derived chromatin (Figs. 6A–6C). At the end of the first division, daughter nuclei of maternal and paternal origin were often physically separated by their respective nuclear lamina (Figs. 6D–6F). In these cases, both paternal and maternal daughter nuclei stayed apposed at the end of mitosis. This result confirmed that in a majority of *mh* embryos, both parental complements entered the second cycle independently. This initial separation of parental complements at the end of the first division was in most cases maintained in subsequent divisions. Indeed, antitubulin staining of *mh* embryos revealed that paternal chromosomes were progressively eliminated from the spindles organized around maternal nuclei (Fig. 7). We observed cycle 3 *mh* embryos in metaphase with the expected four maternal nuclei where paternal chromatin consisted of one or two nuclear bodies. Paternal chromatin either remained partially incorporated in maternal nuclei, a situation presumably leading to aneuploid development, or was excluded from a majority of maternal spindles, leading to haploid development. We observed the former case where each pair of maternal nuclei was bridged with either thin stretches of chromatin or larger chromatin bodies of paternal origin, reflecting an unequal segregation of the paternal complement at the first division (Figs. 7A–7C). In other cases, the paternal nuclei were confined into small, abnormally shaped mitotic spindles (Figs. 7D–7F). Paternal spindles lacked centrosomes or were frequently found to share one centrosome with the closest maternal spindle (Fig. 7E). The *mh* embryo shown in Figs. 7D–7F contained at least three haploid maternal nuclei out of four which were free of "contaminating" paternal chromatin, a situation presumably favorable for haploid development to proceed. Observation of slightly older *mh* embryos confirmed that the division of haploid cleavage nuclei was not impeded by the presence of a small number of aneuploid nuclei (Fig. 8B) and these embryos finally formed a haploid blastoderm with an even repartition of the mitotic spindles (Fig. 8D). The haploid metaphasic spindles appeared narrower than diploid spindles, as described before (Figs. 8C–8D; Komma and Endow, 1995). The rest of *mh* embryos contained only fragmented chromatin or aneuploid nuclei and did not reach the syncytial blastoderm stages (Fig. 8A).

DISCUSSION

We carried out a cytological characterization of fertilization and early development in eggs from *mh Drosophila* females to determine the mechanism by which paternal chromosomes are excluded from haploid nuclei in gynogenetic *mh* embryos.

Our analysis of fertilized eggs from *mh* females did not detect any specific defect in pronuclear formation and migration. The apposed male and female pronuclei entered the first embryonic M phase as in control eggs. Paternal chromosomes were found to congress on the metaphase plate and formed functional centromeres. However, we observed that when maternal sister chromatids began to separate in early anaphase, paternal chromosomes lagged behind on the metaphase plate. Paternal chromatids occasionally adopted an early anaphase configuration with centromeric regions pulled by the spindle but never completed their separation. In telophase, the paternal chromatin stretched between the poles of the spindle and formed a chromatin bridge between the maternal daughter nuclei. At the end of the first division, the bridge ruptured and the paternal complement divided unevenly between the two spindle poles. About a quarter of *mh* embryos completed haploid gynogenetic development despite the presence of a small number of aneuploid nuclei. In other cases, we observed the formation of disorganized chromatin bodies or pyknotic nuclei containing presumably both paternal and maternal chromosomes, a situation leading to early developmental arrest.

The only identified phenotype of paternal chromosomes in *mh* eggs is a defect in separating sister chromatids in anaphase of the first division. A recent study has shown that the absence of histone H3 phosphorylation at serine 10 in a mutant strain of the ciliated protozoa *Tetrahymena thermophila* causes improper chromosome condensation and segregation in mitosis and meiosis (Wei *et al.*, 1999). Interestingly, mutant cells exhibited anaphase bridges and lagging chromosomes that failed to segregate in anaphase. Despite the similarity of our observations in *mh* embryos as those reported for PH3 defective *Tetrahymena* cells, mitotic paternal chromosomes in *mh* eggs contained apparently normal levels of phosphorylated histone H3. According to Wei *et al.* (1999), PH3 is believed to induce a local decondensation of the chromatin and thus help the binding of mitotic chromosomes condensation factors such as SMC proteins (structural maintenance of chromosomes; for reviews, see Nasmyth *et al.*, 2000; Hirano, 1998, 1999; Jessberger *et al.*, 1998; Strunnikov, 1998; Heck, 1997). Thus, we believe that the chromosome condensation machinery is operative both on maternal and on paternal chromosomes in *mh* eggs. However, the state of the paternal chromatin is



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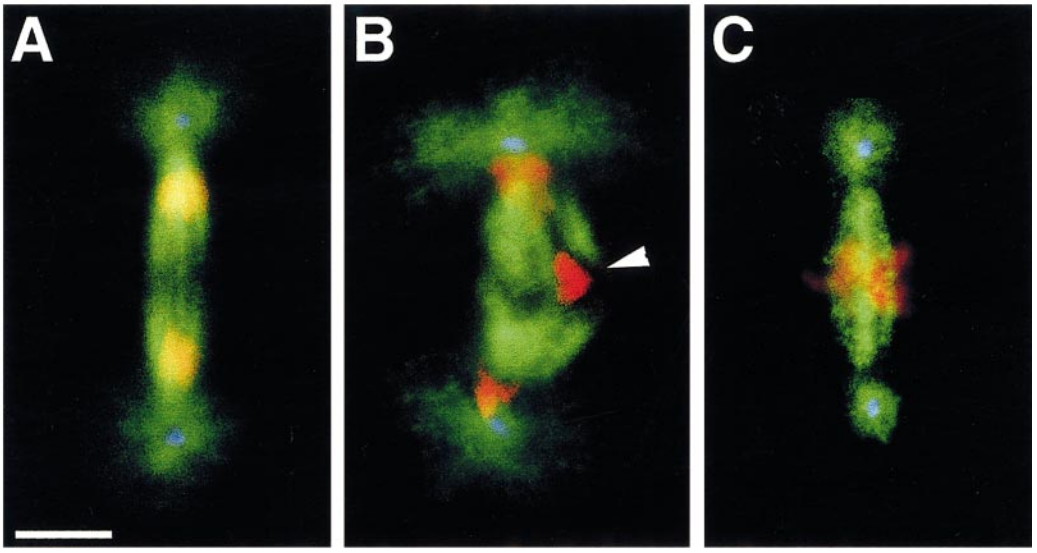

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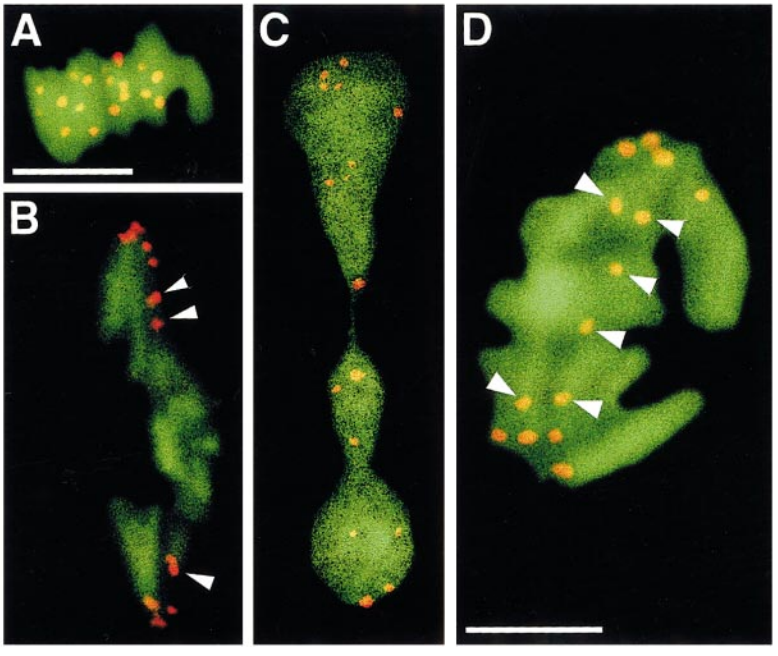

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apparently incompatible with a full separation of sister chromatids.

The gonomeric constitution of the first mitotic spindle and the absence of a true spindle checkpoint in *Drosophila* early embryos (Sullivan *et al.*, 1993) presumably explains why maternal chromosomes in *mh* eggs can complete their division despite the presence of lagging and abnormally dividing paternal chromosomes within the same spindle (see also Callaini *et al.*, 1997). In about 50% of *mh* embryos, the lagging of paternal chromosomes identically reiterated in the second nuclear cycle (see Fig. 2N). At the beginning of the second nuclear division in *mh* embryos, we observed that each parental complement was surrounded by its own nuclear envelope. Each pair of daughter nuclei looked like apposed pronuclei with the exception that the size of the paternal nucleus in each pair was variable, as a consequence of the uneven segregation at the first division. In *Drosophila,* the gonomery at the first division is most probably dependent on the persistence in mitosis of the apposed pronuclear envelopes that keep the parental complements separated until anaphase (Callaini and Riparbelli, 1996). We think that the similar nuclear envelope organization that prevails at the end of the first division in *mh* embryos allows the parental sets of chromosomes to remain separated within each spindle at the second mitosis. In wild-type embryos, both parental genomes share a common nuclear envelope at the end of the first division and subsequent mitoses are apparently not gonomeric. However, little is known about the possible separation of chromosomes according to parental origin in wild-type diploid *Drosophila* cleavage nuclei after the first division. For comparison, the spatial separation of parental genomes is preserved up to the four-cell stage in preimplantation mouse embryos (Mayer *et al.*, 2000) and in human cells (Nagele *et al.*, 1998).

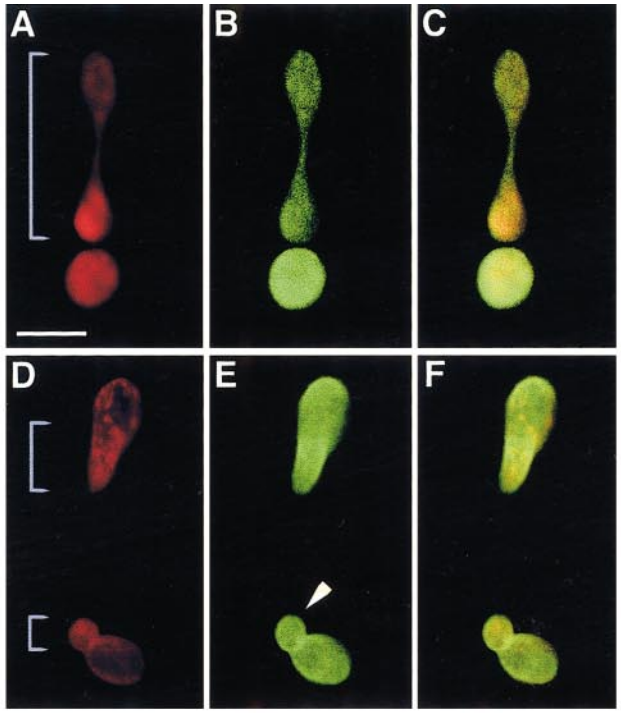
The paternal chromosomes lagging phenotype is still occasionally observed in cycle 3 *mh* embryos but disappears in older stages. We explain this situation by the growing mitotic asynchrony between maternal and paternal genomes as divisions proceed. The *Drosophila* early embryonic divisions are extremely rapid and lack gap phases (Foe *et al.*, 1993). In this context, lagging paternal chromosomes become rapidly out of phase with the pace of the nuclear cycles. Accordingly, we observed that paternal chromosomes were often excluded from maternal spindles by the third embryonic division. The eliminated paternal chromosomes formed abnormal spindles that usually lacked one or both centrosomes. Indeed, we observed that all available centrosomes in *mh* embryos were usually engaged in maternal spindles by the time paternal spindles formed.

Interestingly, a chromatid separation defect similar to that observed in *mh* eggs has also been identified as the mechanism responsible for the elimination of abnormal chromosomes in *Drosophila* embryos (Sullivan *et al.*, 1993) and for the elimination of paternal X chromosomes, as a sex determination mechanism, in syncytial embryos of the dipteran *Sciara coprophila* (de Saint Phalle and Sullivan, 1996). It is possible to envisage the *mh* phenotype as the consequence of the activation of a chromosome elimination process common to early dipteran embryos. Nevertheless, the nature of the effect of *mh* on the paternal chromatin is unknown. The paternal effect mutation *paternal loss* (*pal*) also induces the elimination of paternal chromosomes during *Drosophila* embryonic development (Baker, 1975). In embryos from *pal* fathers, the fourth, X, and Y chromosomes are lost preferably compare to the large second and third chromosomes. According to Baker (1975), the majority of loss occurs during the first three cleavage divisions but more recently paternal chromosome loss has been detected from pronuclear apposition through syncytial blastoderm

FIG. 3. *mh* has no effect on maternal chromosomes divisions. Confocal section showing the anaphase of the first mitosis in an egg laid by a double-mutant ssm^{185b} *mh* female and stained for DNA. The condensed nucleus on the left is the male pronucleus which clearly displays the *ssm* phenotype (Loppin *et al.*, 2000). With the exception of the small fourth chromosome which was out of this section, all maternal chromatids can be seen to separate successfully at anaphase. Bar: 5 μm.

FIG. 4. Structure of the gonomeric spindle in *mh* eggs. Eggs were stained for DNA (red), microtubules (green), and centrosomes (blue). (A) A wild-type first embryonic spindle in late anaphase. (B) An *mh* egg with its first spindle containing maternally derived chromatids in late anaphase and lagging paternal chromatin (arrowhead). Gentle pressure was applied on the preparation in (B) to reveal each bundle of microtubules constituting the gonomeric spindle. (C) A bipolar first spindle in a *KLP3A mh* fertilized egg containing only the paternal chromosomes. Bar: 10 μm.

FIG. 5. Paternal chromosomes form functional centromeres in *mh* embryos. Cycle 1 (A, C) and cycle 2 (B, D) *mh* embryos were stained for DNA (green) and for the pericentromeric Cid protein (red). In metaphase of the first division, both haploid sets of parental chromosomes contained well-defined centromeres (A). In anaphase of the first or second divisions, paternal chromatids lagged behind maternal chromatids despite the presence of centromeres oriented poleward (arrowheads in B). In telophase of the first division, the four maternal centromeres were usually found at the tip of the decondensing maternal daughter nuclei (clearly visible in the upper nucleus), whereas paternal centromeres were positioned at random throughout the stretched paternal chromatin (C). The nucleus represented in (D) is in early anaphase of the second mitosis and all centromeres are clearly identified in this projection of optical sections. The four pairs of maternal centromeres are normally positioned at the tip of the chromatids and paternal centromeres (arrowheads) lag behind as in (B). Interestingly, a pair of paternal centromeres is clearly missing in this nucleus, suggesting that a paternal chromosome was lost at the first division. Bar in A represents 10 μm for (A–C); bar in D represents 5 μm.



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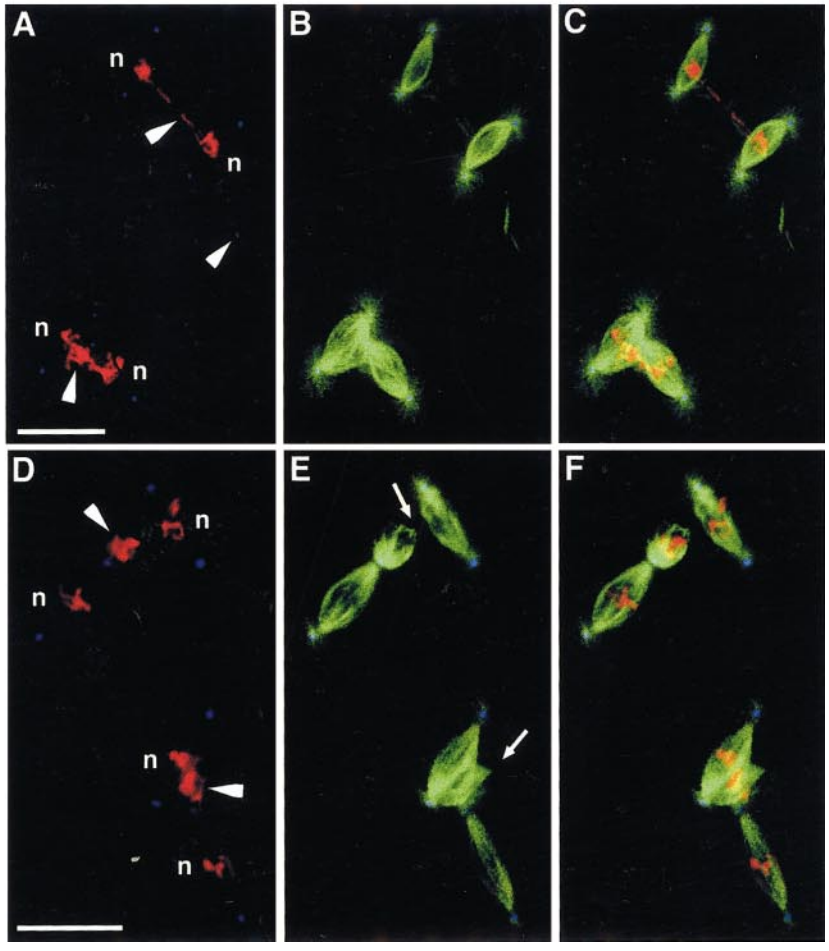

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stages (reviewed in Fitch *et al.*, 1998). The *pal* phenotype is thus clearly different from the *mh* phenotype where all paternal chromosomes are affected at the first division. Important clues come from the comparison of the *mh* phenotype with the cytological defects induced by another paternal effect mutation called *ms(3)K81* (Fuyama, 1984; Yasuda *et al.*, 1995). In this case, paternal chromosomes are unable to divide in anaphase of the first division (Yasuda *et al.*, 1995; Loppin *et al.*, 2000). A small fraction of *K81* embryos (<10%, Yasuda *et al.*, 1995) develop as haploid gynogenetic embryos but the majority arrest their development after a few nuclear divisions and contain pyknotic nuclei. The presumed absence of expression of the *K81* gene product during spermatogenesis is apparently responsible for the inability of the male pronucleus to divide properly after fertilization. The K81 protein has not yet been identified but it could function in allowing the sperm chromatin to remodel properly once liberated in the egg cytoplasm (Yasuda *et al.*, 1995). The *mh* phenotype suggests that this process is also under the control of maternal products deposited in the oocyte. In both *mh* and *K81* phenotypes, the decondensation of the sperm chromatin is completed and chromosomes align on the metaphase plate (this study; Loppin *et al.*, 2000; Yasuda *et al.*, 1995). Thus we can speculate that either sperm chromatin remodeling, DNA replication, or chromosome condensation is affected in these mutants. In this regard, incompletely replicated or damaged DNA affects the segregation of sister chromatids in syncytial cycles of *Drosophila* embryos (Fogarty *et al.*, 1997). Our present analysis suggests that the male pronucleus replicates its DNA in *mh* eggs as shown by the PH3 staining and the formation of paternal sister chromatids. However, we cannot exclude the presence of nonreplicated regions of the male genome or nonrepaired double-strand breaks undetectable at this level of observation.

A combination of paternal and maternal effects controlling the ability of paternal chromosomes to participate in embryonic development also underlies the phenomenon of cytoplasmic incompatibility (CI). CI is one particular aspect of a widespread reproductive parasitism in arthropods induced by bacterial endosymbionts of the genus *Wolbachia* (Breeuwer and Werren, 1990; Boyle *et al.*, 1993; Reed and Werren, 1995; Karr, 1996). In *Drosophila*, CI occurs in eggs produced from crosses between males whose germlines are infected by *Wolbachia* and noninfected females. In *D. simulans*, CI usually induces a strong embryonic lethality (>95%) and only a few viable adults are recovered from incompatible crosses (Hoffmann *et al.*, 1986; Boyle *et al.*, 1993; Poinsot *et al.*, 1998). Recent cytological studies of CI in *D. simulans* have revealed a delay of paternal chromosome condensation and a defect of chromatid separation in anaphase of the first embryonic mitosis (Lassy and Karr, 1996; Callaini *et al.*, 1997), a situation similar to the phenotype of *K81* embryos (as mentioned in Karr, 1996) and of *mh* embryos. Current models suggest that an epigenetic modification of the sperm chromatin during spermatogenesis could be responsible for the paternal chromosomes defects in embryos from incompatible crosses (Bourtzis *et al.*, 1998; Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Presgraves, 2000). This model implies that, in compatible crosses between infected males and females, the bacteria present in the egg cytoplasm can rescue the sperm chromatin modification that occurred during male gametogenesis. CI has usually a weaker effect in *D. melanogaster* than in *D. simulans*. However, this is probably a consequence of the small percentage (8%) of germline cysts infected by the bacteria in the testes of adult *D. melanogaster* males (more than 80% of cysts are infected in *D. simulans;* Poinsot *et al.*, 1998).

The striking phenotypic similarities between *mh-*, *K81-*, and CI-derived embryos suggest that a common step in the process of sperm nucleus transformation is affected, respectively, by maternal, paternal, and bacterial controls. Emerging data brought by genetics of fertilization in *Drosophila* illustrate the complex cooperation of the egg cytoplasm and

FIG. 6. Nuclear lamina reformation in late mitosis of cycle 1 *mh* embryos. Confocal images of cycle 1 *mh* embryos stained for DNA (red; A–D) and lamin Dm_0 (green; B, E). (C, F) are merged views. (A–C) is an embryo in telophase showing its bridge of paternal chromatin surrounded by its own nuclear lamina. Only one maternal daughter nucleus is seen in this field (round nuclei at the bottom), also wrapped in its own envelope. (D–F) Another *mh* embryo in late telophase showing the unequally segregated paternal chromatin apposed to the maternal nuclei. Again, nuclear lamina separate both types of chromatin (arrowhead in E). Blue brackets in A and D indicate the position of lagging paternal chromatin. Bar represents 10 μm for all panels.

FIG. 7. Mitotic spindle organization in early *mh* embryos. Panels (A–C) and (D–F) show two different *mh* embryos in metaphase of nuclear cycle 3 stained for DNA (red; A, C, D, F), microtubules (green; B, C, E, F), and centrosomes (blue; all). The four haploid sets of maternal chromosomes are indicated with the letter (n) and the paternal chromatin is identified by arrowheads in (A, D). The upper embryo (A–C) contains two pairs of maternal nuclei (each pair represents the daughter nuclei of the second division). The lower pair incorporated disorganized chromatin bodies of paternal origin (lower arrowhead). The corresponding spindles contain both maternal and paternal chromatin and have fused together (B, C). A thin paternal chromatin bridge has formed between the maternal nuclei of the upper pair (upper arrowhead in A). This bridge is associated with a few microtubules (B, C). Fragments of paternal chromatin have also dispersed in this embryo (middle arrowhead in A). (D–E) In contrast to the embryo in (A–C), in the lower embryo the paternal chromatin separated in two sets of approximately equal DNA content during the first division (arrowheads in D). Each paternal nucleus is contained in a small, abnormal spindle that lacks a centrosome (arrows in E) and shares the other with a maternal spindle. Both embryos presented in this figure contain a normal triploid polar body (not shown). Bars: 20 μm.

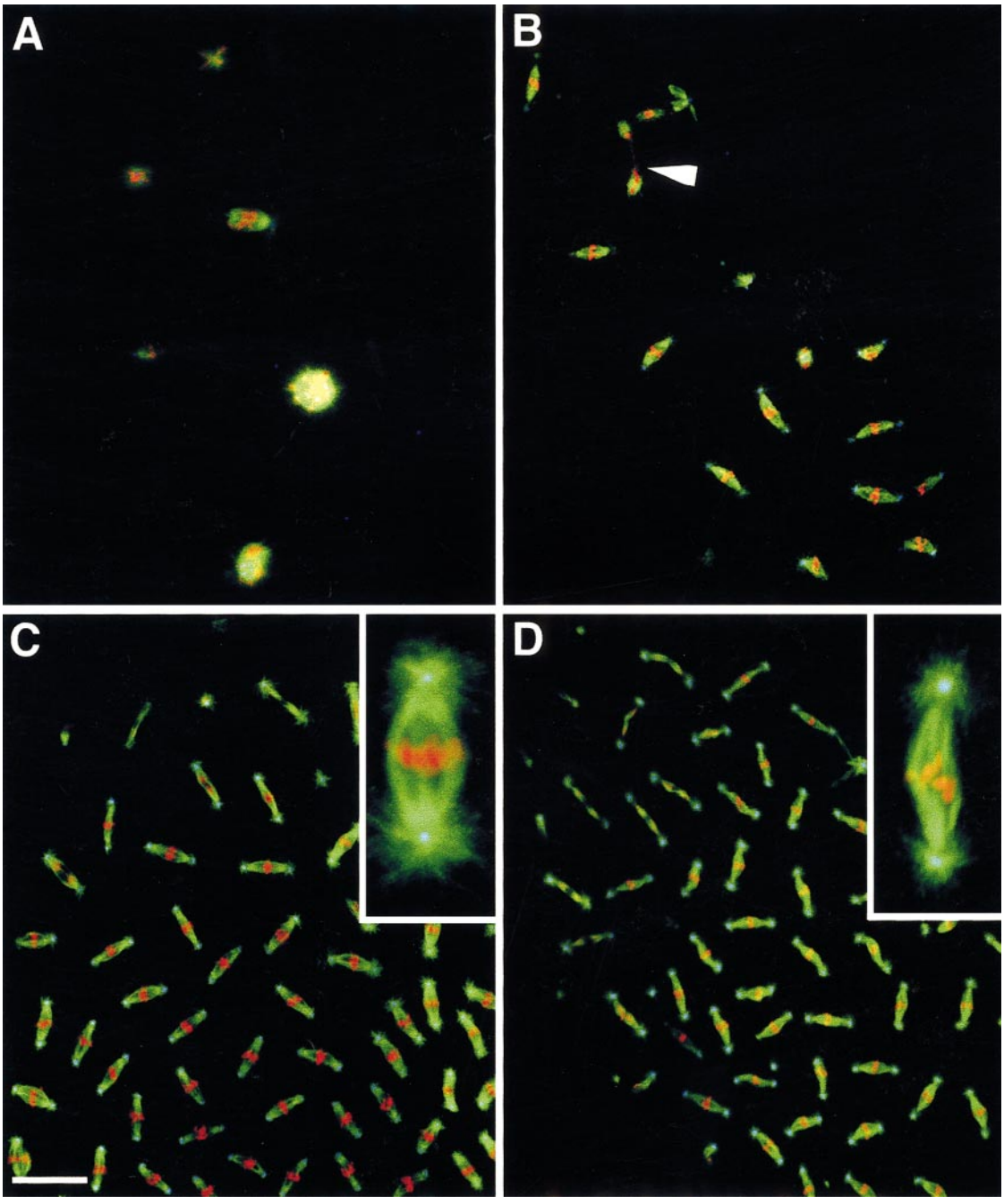

FIG. 8. Early arrest phenotype versus haploid development of *mh* embryos. Wild-type (C) or *mh* embryos (A, B, D) stained as in Figs. 6 and 7. (A) is an aneuploid *mh* embryo which contains only aneuploid nuclei of various size and aspect associated with mostly disorganized microtubules. (B) A cycle 5 *mh* embryo containing a majority of maternal haploid spindles. A few abnormal nuclei are present (arrowhead). (C) A wild-type cycle 9 syncytial blastoderm embryo with diploid metaphasic nuclei disposed in a cortical monolayer. (D) A haploid *mh* embryo at the same stage presenting an even repartition of the spindles. The haploid spindles are narrower than control diploid spindles (insets in C and D). Bar represents 20 μm for all.

the male gamete in controlling coordination of parental chromosomes to form a diploid zygote.

The *mh* mutation was previously mapped in the *v–g* region on the X chromosome (Debec, 1978). Since *mh* is not covered by any available deletion (see Materials and Methods), our phenotypic characterization relies on the analysis

of eggs produced by homozygous females. Even if it is improbable, we cannot formally exclude the possibility that the paternal chromosome elimination phenotype we observed is not dependent on the presence of another tightly linked mutation. In order to definitely map the *mh* locus and confirm the present analysis, new tagged *mh* alleles will be required.

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