Toward a Comprehensive Genetic Analysis of Male Fertility in *Drosophila melanogaster*

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Manuscript received November 3, 2003
Accepted for publication January 14, 2004

ABSTRACT

Drosophila melanogaster is a widely used model organism for genetic dissection of developmental processes. To exploit its full potential for studying the genetic basis of male fertility, we performed a large-scale screen for male-sterile (*ms*) mutations. From a collection of 12,326 strains carrying ethyl-methanesulfonate-treated, homozygous viable second or third chromosomes, 2216 *ms* lines were identified, constituting the largest collection of *ms* mutations described to date for any organism. Over 2000 lines were cytologically characterized and, of these, 81% failed during spermatogenesis while 19% manifested postspermatogenic processes. Of the phenotypic categories used to classify the mutants, the largest groups were those that showed visible defects in meiotic chromosome segregation or cytokinesis and those that failed in sperm individualization. We also identified 62 fertile or subfertile lines that showed high levels of chromosome loss due to abnormal mitotic or meiotic chromosome transmission in the male germ line or due to paternal chromosome loss in the early embryo. We argue that the majority of autosomal genes that function in male fertility in Drosophila are represented by one or more alleles in the *ms* collection. Given the conservation of molecular mechanisms underlying important cellular processes, analysis of these mutations should provide insight into the genetic networks that control male fertility in Drosophila and other organisms, including humans.

MALE fertility depends on the proper function of many developmental and physiological processes, beginning with germ cell determination and ending with the promotion of normal development of the fertilized ovum by paternal contributions. Intervening between these steps are stem cell function, spermatogenesis (including gonial proliferation, spermatocyte growth, meiosis, and spermiogenesis), sperm transfer and storage, and fertilization. Male-sterile mutations can interrupt any step in this progression and therefore provide excellent material for investigating its genetic control. The ideal system for generating and characterizing male-sterile mutations is *Drosophila melanogaster*. In Drosophila, the adult testis is a single tube in which the developmental stages are laid out in chronological order from the stem cells at the tip to mature spermatozooa at the base, with particular stages occurring at specific regions along its length. It is a steady-state system with primary gonial cells being generated from stem cells at the tip and 64 mature sperm per primary gonial cell being transferred into the seminal vesicles for export at the base. Moreover, Drosophila have prototypical, flagellated sperm. Both the morphology and dynamics of spermatogenesis in this organism have been extensively characterized, facilitating the identification of mutational lesions affecting specific aspects of male fertility (Tates 1971; Tokuyasu 1974a,b, 1975a,b; Tokuyasu *et al.* 1972, 1977; Lindsley and Tokuyasu 1980; Fuller 1993). Because the detailed features of this developmental program appear to be well conserved among phylogenetically diverse animal groups, discoveries made in the fly system will have broad applicability to other species, including our own (Hackstein *et al.* 2000).

Flies are convenient organisms for mutagenic treatment, and selective screens for male-sterile mutations are highly efficient. Several modest screens have been carried out in the past, using ethyl methanesulfonate (EMS; Hackstein 1991), *P* elements (Cooley *et al.* 1988; Berg and Spradling 1991; Castrillon *et al.* 1993), or X rays as the mutagenic agent. We considered it useful to perform an exhaustive screen to identify as many of the genes involved in assuring male fertility as possible. The goal was to allow the recovery of male-sterile mutations and paternal-effect mutations that previous studies had shown to be relatively rare and understudied. Accordingly, we have screened a collection of >12,000 lines that carry in balanced condition a heavily mutagenized autosome observed to survive in homozygous condition (see Koundakjian *et al.* 2004, accompanying article in this issue). Here we report the identifi-

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cation and initial classification of a collection of >2000 male-sterile mutant strains.

The scope of our screen was such that we argue that we have mutated the vast majority of the second- and third-chromosome genes whose primary function is related to male fertility and recovered rare male-sterile alleles of many other genes that function primarily in other processes. We have classified the mutations into several morphologically recognizable groups on the basis of initial cytological studies. Although more extensive work is required to determine the number of complementation groups and the identity of genes represented by the male-sterile mutations, this collection, which is currently maintained in the laboratory of Charles Zuker, represents an exceedingly valuable resource for the study of the genetic control of spermatogenesis as well as other features of male reproduction. As these mutations are characterized genetically, phenotypically, and molecularly, the network of gene activity controlling male fertility will assuredly emerge. Such information should be highly useful in understanding the genetic causes of male sterility in humans; ~5% of men are infertile, presumably owing to genetic lesions in many instances (De Kretser and Baker 1999; Hackstein *et al.* 2000).

In addition to screening for male-sterile lines, we designed the screen to enable us to follow the inheritance of the paternal fourth chromosome in the fertile lines. We recovered 62 instances of mutations leading to the loss of chromosome 4. Similar searches for autosomal mutations affecting chromosome transmission in males were carried out in the past by Sandler (1971), Sandler *et al.* (1968), and Ivy (1981); however, these reports deal with fewer than a dozen mutations, nearly all of which have been lost.

MATERIALS AND METHODS

Identification of male-sterile and chromosome loss lines: As described in the accompanying article, Koundakjian *et al.* (2004) examined >72,000 F_3 cultures carrying EMS-treated second or third chromosomes and retained 12,336 lines in which the mutagenized chromosome was homozygous viable. There are 6079 lines, designated Z2-0001–Z2-6079, which carry mutagenized second chromosomes marked with *cn* and *bw* and balanced over *CyO* and 6257 third-chromosome lines, designated Z3-0001–Z3-6257, which are homozygous for a second chromosome marked with *bw* and carry a mutagenized third chromosome marked with *st* and balanced over *TM6B-Tb*. Although each Z line was originally selected by Koundakjian *et al.* (2004) as yielding a reasonable frequency of homozygous white-eyed *cn bw* or *bw; st* flies among balancer heterozygotes, 9% of the collection (746 second-chromosome lines and 365 third-chromosome lines) were no longer doing so at the time we screened the collection. For those lines that produced homozygotes, the yield was highly variable. We were therefore able to screen 93% of the Zuker collection (5398 second-chromosome lines and 6104 third-chromosome lines, a total of 11,502 lines) for male fertility.

We crossed homozygous males from each of the Z strains to females carrying marked X and fourth chromosomes. In general, three to six *cn bw* or *bw; st* males were crossed to two to three *y w sn*^{3}*; C(4)RM, ci ey*^{R}*/0* females in a vial. The crosses were maintained at room temperature and fertility was monitored after 5 or more days. For fully fertile crosses, the progeny yield was typically >100 individuals. The lines in which homozygous males failed to produce progeny were classified as male sterile. For the putative male-sterile lines, we either retested for fertility in a second cross or performed a cytological analysis as described below to verify sterility. We also retained a set of 174 third-chromosome lines that produced 10 or fewer individuals in a vial cross; these lines were classified as "barely fertile."

To recover mutations inducing chromosome loss, we scored the progeny of each fertile cross for somatic loss of the paternal X chromosome, evident as patches of y (yellow body color), w (white eye facets), and sn (singed bristles) in daughters. The use of the compound fourth chromosome, *C(4)RM, ci ey*^{R}, in the maternal genotype allowed us to monitor for loss of the paternal fourth chromosome by the presence of offspring with ci (wing vein interrupted) and ey (eyeless) phenotypes. In practice, only three cases of X chromosome mosaicism were observed among thousands of crosses scored, compared to hundreds of cases of sons and daughters that were nullosomic or mosaic for the paternal fourth chromosome. The compound fourth chromosome permitted the recovery of the exceptional offspring as diplo-4, rather than as haplo-4 flies, which show reduced viability and delayed eclosion.

We recovered several hundred lines that showed one or more exceptional offspring in the fertility test with a higher number observed for the Z3 collection compared to the Z2 collection. Sixty-two lines were confirmed as reliable chromosome loss lines, either producing ≥5% nullosomic or mosaic progeny in three separate trials or showing clear cytological evidence for chromosome loss during spermatogenesis. These lines constitute the male chromosome loss (*mcl*) collection. To establish allelism, the *mcl* lines were crossed *inter se* or with strains carrying known chromosome loss mutations, including *pal*^{1} (Baker 1975) and selected meiotic mutations (Sandler 1971; Lindsley and Zimm 1992), in this study and in the laboratories of B. McKee, J. Tomkiel, K. McKim, and S. Hawley.

We tested 81% of the 2458 male-sterile, barely fertile, and chromosome loss lines for female fertility. Typically, two or more homozygous females were placed in a vial with their heterozygous brothers and fertility was scored several days later, but occasionally only a single female could be tested. As we did not quantify progeny yields in these crosses, these data are considered for a preliminary assessment of the proportion of lines that were both male and female sterile. In some cases, we were also able to record the nature of female sterility with respect to whether or not eggs were produced. In addition, in the process of selecting males for fertility tests, other visible phenotypes were noted, *e.g.*, body color, wing phenotypes, bristle number and shape, and eye structure. Eye-color mutations were undetectable, owing to the white-eyed phenotype of the homozygotes. We also identified nine Z2 strains that yielded homozygous females but not homozygous males. Complementation analyses showed that each carried a mutation in a known male-specific lethal gene. The alleles were designated *msl-1*^{Z2681}, *msl-1*^{Z5150}, *msl-1*^{Z5823}, *msl-2*^{Z2397}, *msl-2*^{Z2421}, *msl-2*^{Z3890}, *mle*^{Z1536}, *mle*^{Z3543}, and *mle*^{Z5317}.

Cytology: To classify the *ms* mutant lines, we examined two or more homozygous males for defects in spermatogenesis using a simple cytological assay. Adult males were dissected in Hoyle's medium (Baccetti *et al.* 1979). The testes were isolated and transferred to a small drop of saline on a slide. Each testis was nicked at several places along its length to release cells and the preparation was covered with a coverslip.

Excess liquid was wicked off at the edge of the coverslip using absorbant paper to flatten the preparation. Preparations were examined at ×100–×400 magnification using phase-contrast optics to assess the overall appearance of the testis and seminal vesicle and the presence and morphology of stages of spermatogenesis, specifically gonial cells, primary spermatocytes, onion-stage spermatids, elongating spermatids, and mature sperm. The seminal vesicles were monitored for the presence of sperm, and sperm activity was characterized as immotile, weakly motile, or motile. Images were captured using a Nikon photomicroscope equipped with a CoolSNAP cool-charged coupled device camera (RS Photometrics) and images were processed using Adobe Photoshop 7.0 software (Adobe Systems).

Nomenclature and database information: Mutations are described in Lindsley and Zimm (1992) and FlyBase (http://flybase.bio.indiana.edu/genes/). As described by Koundakjian *et al.* (2004, accompanying article), the Zuker lines are designated by their Z number. The male-sterile lines also carry the *ms* designation with the second- or third-chromosome identity indicated, *e.g.*, *ms(2)Z0758* or *ms(3)Z1870*. We have temporarily designated the male chromosome loss lines as *mcl*, *e.g.*, *mcl(2)Z0552* or *mcl(3)Z2094*. When more is known about the mutation, for example, its allelism to a known gene or its cytogenetic location, the Zuker line designation appears in the superscript, as in the case of twe^{Z0758}, which was found to be an allele of *twine* by Maines and Wasserman (1999), or, for example, *ms(2)Z0103*, which was renamed as $ms(2)34F^{Z0103}$. In some cases, the *ms* mutation was found to be a viable allele of an essential gene; for example, *ms(2)Z4556* was renamed $l(2)35F^{msZ4556}$.

The Z lines are currently being maintained in the laboratory of Charles Zuker at the University of California-San Diego and are available upon request (czuker@ucsd.edu). Our observations on the male-sterile and chromosome loss lines are recorded in a searchable database using FileMaker Pro 6.0 software (Deneba). The database is available from the authors upon request.

RESULTS

A large-scale screen for male-sterile and paternal chromosome loss mutants: The initial goal of our study was to isolate mutations affecting sperm function and paternal effects on early embryogenesis. Our previous studies (Fitch *et al.* 1998) and those of other investigators (Hackstein 1991; Castrillon *et al.* 1993) indicated that these classes of mutations were relatively rare among the many other types of mutations affecting male fertility. We were therefore interested in performing a large-scale screen for male sterility and paternal-effect mutations, including those resulting in paternal chromosome loss in the early embryo. A summary of the scope of the screen is shown in Table 1. We were able to screen for mutations causing male sterility or male chromosome loss in 11,502 of the 12,336 Z lines described above. We also classified a set of lines from the third-chromosome screen as barely fertile since they produced <5% of the number of progeny typically seen in fertile crosses.

Phenotypic classification of the male-sterile lines: To determine whether the male sterility of each line was associated with an observable defect during spermatogenesis, we performed a cytological analysis of the adult testis using a saline squash technique. Figure 1 compares micrographs of stages of spermatogenesis in wild-type males (left) and mutant lines (right) to illustrate representative defects. We classified the mutants into six general phenotypic groups on the basis of whether they showed: (1) agametic gonads; (2) gonial or young spermatocyte arrest; (3) mature spermatocyte arrest or abnormalities; (4) onion-stage spermatid abnormalities; (5) defects in spermatid elongation, coiling, or individualization; or (6) mature sperm. The relative frequencies of the six phenotypic classes are shown in Table 2.

TABLE 1

Summary of the screen for male-sterile and male chromosome loss lines

	n	%
Chromosome 2 lines screened	5,398	
Fertile	4,403	
Barely fertile	6	
Sterile	962	17.8
Chromosome loss	21	0.4
Chromosome 3 lines screened	6,104	
Fertile	4,634	
Barely fertile	174	
Sterile	1,254	20.5
Chromosome loss	41	0.7
Total lines screened	11,502	
Sterile	2,216	19.3
Chromosome loss	62	0.5

Relatively few lines were in the first three categories, which represent defects in early stages of spermatogenesis. Sixty-six lines were classified as agametic because they had greatly reduced testes that were either extraordinarily thin or, in extreme cases, comparable in size to that of the seminal vesicles with minimal cellular content of uncertain nature. This class should include mutants that fail to specify or maintain a germline. Early arrest mutants do not appear to progress far beyond the proliferative mitotic stages and show varying amounts of gonial hyperplasia or accumulation of young primary spermatocytes (Figure 1D). Initial studies revealed that the 40 mutations in this class include six *benign gonial cell neoplasia* (*bcgn*), three *traffic jam* (*tj*), and two *bag-of-marbles* (*bam*) alleles (data provided by M. T. Fuller and D. McKearin, personal communication). Sixty-seven mutant lines accumulated primary spermatocytes at later stages of maturity, with reduced numbers or no postmeiotic stages recognizable. This class included new alleles of many of the previously identified spermatocyte arrest genes (see *e.g.*, White-Cooper *et al.* 2000; Hiller *et al.* 2001).

The remaining three classes comprised 92% of the collection and showed defects that occur during or after

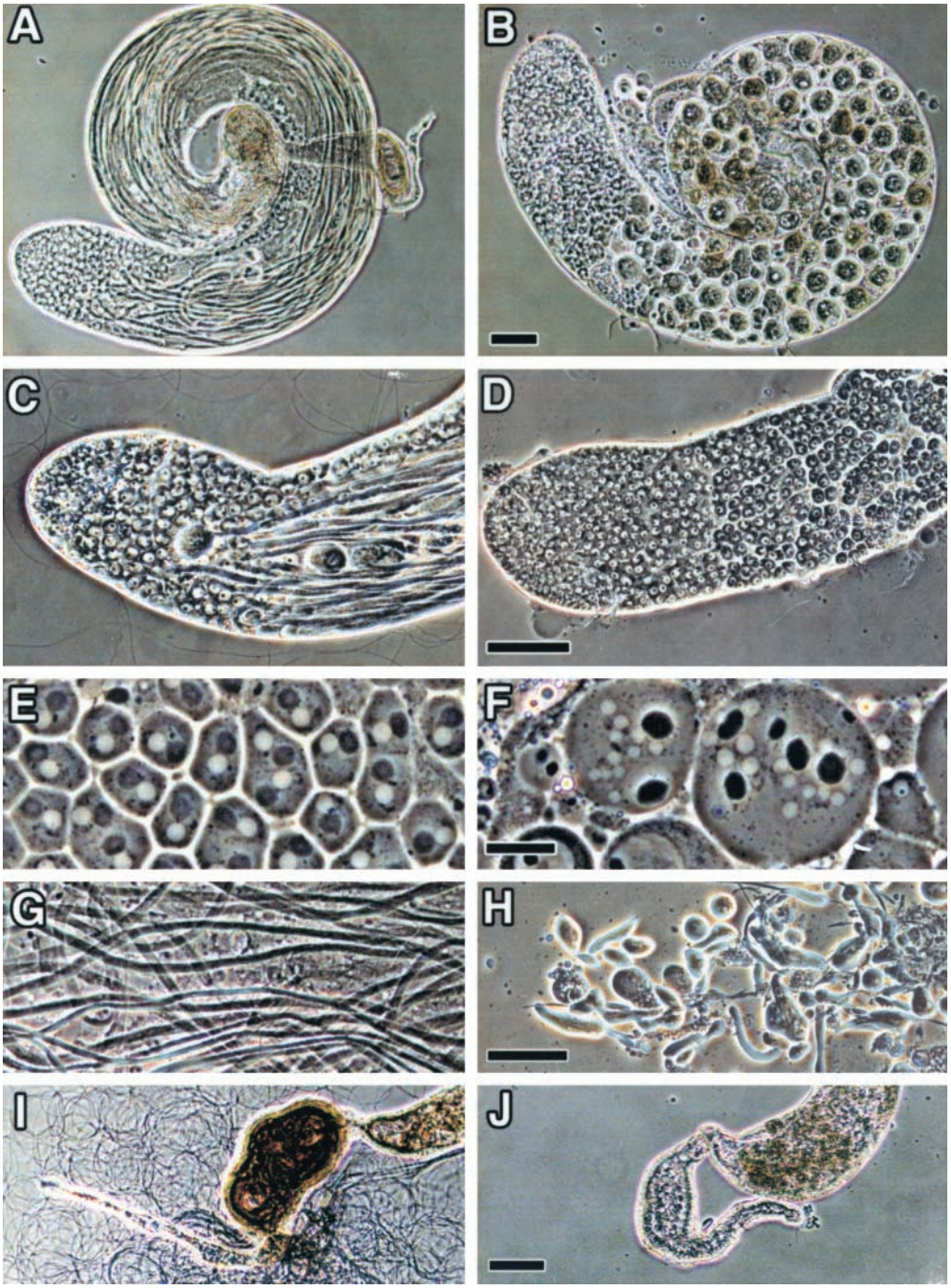

FIGURE 1.—Comparisons of the stages of spermatogenesis in normal *bw; st* adult males (left) and *ms* mutants (right) as viewed with phase-contrast microscopy. The low magnification view of the normal testis in A shows the overall distribution of stages. Higher magnification views in C, E, G, and I show successively later stages. At the apical tip of the testes (A and C), the small gonial cells undergo four mitoses, with each yielding a cyst of 16 cells that remain interconnected through cytoplasmic bridges. Growing primary spermatocytes range in size but are prominent in C due to their round white nuclei and dark dot-like nucleolus. Meiotic cysts are present along the inner curve of the testis (A). (E) A portion of a postmeiotic cyst that contains 64 onion-stage spermatids; nuclei (white) and their adjacent nebenkerns (dark gray) are present in a 1:1 ratio and are equivalent in size. A few of the cytoplasmic bridges were broken in this preparation, revealing the cytoplasmic continuity between spermatids. Differentiating cysts occupy a large proportion of the testis to accommodate the elongating bundles of sperm tails (G). At the base of the testis is the sac-like seminal vesicle that is filled with sperm in A and I. The vesicle has been punctured in I to allow the release of motile sperm. Corresponding stages of spermatogenesis in mutant strains are shown at the right. In B, the testis of *ms(2)Z0738* males shows an accumulation of cysts at the primary spermatocyte stage. Only a few cysts appear to enter meiosis, and all cysts show some differentiation, but fail to elongate. In D, about three-quarters of the highly reduced testis of *ms(3)Z2995* is shown. The testis is occupied by gonial cells and early primary spermatocytes and lacks later stages. In F, onion-stage cysts of *ms(3)Z4934* show prevalent micro- and macro-spermatid nuclei and macro-nebenkerns, indicating defective meiotic chromosome segregation and cytokinesis. In H, the cysts of *ms(3)Z2420* are irregularly shaped, indicating variable arrest during elongation. In J, the hallmark of the "classic" ms phenotype is shown for *ms(2)Z2181.* Degenerating sperm bundles are present at the base of the testis and the seminal vesicle is devoid of sperm. Bars apply to both the left and right of each row and indicate 100 μm but 20 μm for E and F.

meiosis. The abnormal onion-stage spermatid class accounted for 19%, or 416 lines. Spermatid nuclei and nebenkerns, which form postmeiotically from the fusion of secondary spermatocyte mitochondria, are normally present in equal numbers and are of the same diameter at the onion stage, reflecting the even allocation of chromosomes and cytoplasmic material to the four products of meiosis (Figure 1E). The mutant lines had

TABLE 2

Classification of the phenotypes of male-sterile lines

Class	*n*	%
Agametic	66	3.1
Gonial arrest	40	1.9
Spermatocyte defective	67	3.1
Irregular onion-stage spermatids	416	19.5
Elongation, coiling, or individualization defective	1138	53.4
Mature sperm produced	404	19.0
All lines classified cytologically	2131	

unequal numbers, sizes, or morphology of the nuclei and the nebenkerns among spermatids developing synchronously within a cyst. Nebenkern defects included misshapen or vacuolated forms. In some mutants, there were micro- and macronuclei, indicative of abnormal chromosome segregation (Hardy 1975). A common defect observed was the "four-wheel-drive phenotype" (Brill *et al.* 2000) in which a single large nebenkern was associated with four regularly sized nuclei. This phenotype is diagnostic of mutations affecting spindle assembly and cytokinesis (Bucciarelli *et al.* 2003; Farkas *et al.* 2003). Recent studies have shown that 34 mutations in this class define 19 complementation groups (Giansanti *et al.* 2004). The abnormal phenotype of onion-stage spermatids in a mutant defective in both chromosome segregation and cytokinesis is shown in Figure 1F.

Defects occurring during the postmeiotic stages of spermatogenesis were the most common phenotypes observed in the male-sterile lines and were characteristic of many lines that showed morphological defects in earlier stages. However, 1138 lines, which constitute 53% of the *ms* collection, appeared morphologically normal at all prespermiogenic stages, but failed during spermatid differentiation. In a few cases, spermatid elongation began but arrested early or midway through the process, producing oval- or teardrop-shaped cysts (Figure 1H). Most commonly, elongation was extensive or complete and long bundles of differentiated spermatids were abundant; however, sperm individualization did not occur and there was no coiling. Instead the base of the testis was filled with debris and the seminal vesicles remained empty (Figure 1J). This mutant phenotype has been described as the "classic" male-sterile phenotype (R. W. Hardy and D. L. Lindsley, unpublished results; see *e.g.*, Fuller 1993) because it is so common. The large number of these mutants indicates that the final steps of differentiation that culminate in sperm individualization may be the most sensitive of all steps of spermatogenesis to disruptions.

In the sixth group of mutant lines, representing 19% of the collection, the seminal vesicles contained sperm. In many cases the seminal vesicles were as engorged with sperm as they are in wild-type males (Figure 1I). A small number of the mutants in this class produced sperm that appeared immotile; most produced reasonable quantities of motile sperm. This mature sperm class included mutants that were sterile due to defects in mating behavior, sperm transfer to or storage in females, or in fertilization. This class also included paternal-effect lethal mutations, which we define as mutants in which the sperm entered the egg but failed to support normal embryogenesis (Fitch *et al.* 1998).

For a small subset of the *ms* lines, homozygous adult males could no longer be recovered from the balanced stocks by the time the cytological assays were performed. This reduced the number of strains that we were able to cytologically classify to 2131 lines, or 96% of the Z collection.

On the relationship between male and female fertility: To determine the proportion of male-sterile lines that were also female sterile, we tested the fertility of females homozygous for the *ms* chromosome for 1899 lines. Overall, 40% of these lines were also female sterile. Further genetic analyses are required to determine which cases are due to a single mutation affecting fertility in both sexes and which are due to two separable mutations on the Z2 or Z3 chromosome. *A priori*, we expect there to be common genetic controls for the early stages of germ cell development in males and females; however, given the striking morphological differences in oogenesis and spermatogenesis at later stages, we expect a larger proportion of sex-specific genetic controls of processes from the primary gametocyte on. Consistent with this idea, we found that 71% of 102 *ms* chromosomes carrying mutations that resulted in agametic or gonial arrest phenotypes were female sterile, whereas only 38% of the remaining 1797 in the other four categories were female sterile.

Classification of the male chromosome loss lines: We incorporated recessive maternal markers in our screen to identify lines in which homozygous mutant males were fertile but produced progeny that had lost the paternal fourth chromosome. We recovered 21 second-chromosome lines and 41 third-chromosome lines in which ≥5% of the progeny produced were nullosomic or mosaic for the paternal fourth chromosome. These lines, referred to as the male chromosome loss or *mcl* lines, have retained the chromosome-loss phenotypes after several years, with over half exhibiting chromosome-loss rates as high as 20–50% (Table 3). Progeny yields were highly variable among the lines. Some lines were barely fertile, as might be expected if loss of the major autosomes was occurring at a high rate, resulting in the production of nonviable aneuploid embryos.

Cytological and genetic studies permitted a classification of the *mcl* lines according to stage of chromosome loss and showed that premeiotic, meiotic, and postfertilization chromosome-loss mutants were recovered in our screen. Two mutations, which defined a single comple-

TABLE 3

Classification of the chromosome loss lines

Time of chromosome loss	Locus	Line (% chromosome 4 loss)[a]	Reference
Premeiotic	*nuclear blebber*	nbl^{Z1837} (14), nbl^{Z4522} (30)	This study
Meiotic	*teflon*	tef^{Z1869} (16), tef^{Z3455} (27), tef^{Z4169} (14), tef^{Z5549} (6), tef^{Z5864} (7)	Tomkiel *et al.* (2001)
	ord	ord^{Z5736} (21)	B. McKee (personal communication)
Meiotic[b]	ND	*mcl(2)Z0198* (25), *mcl(2)Z0218* (13), *mcl(2)Z0338* (35), *mcl(2)Z1525* (7), *mcl(2)Z3384* (5), *mcl(2)Z3534* (15), *mcl(2)Z4579* (5), *mcl(3)Z0317* (32), *mcl(3)Z0589* (20), *mcl(3)Z0777* (5), *mcl(3)Z1483* (10), *mcl(3)Z1492* (5), *mcl(3)Z1550* (33), *mcl(3)Z1641* (19), *mcl(3)Z1898*, *mcl(3)Z1956* (21), *mcl(3)Z2086*, *mcl(3)Z2094*, *mcl(3)Z2138* (30), *mcl(3)Z2761* (27), *mcl(3)Z3298* (35), *mcl(3)Z3320* (47), *mcl(3)Z3370* (19), *mcl(3)Z3401* (7), *mcl(3)Z3426* (22), *mcl(3)Z3822* (50), *mcl(3)Z4085* (27), *mcl(3)Z4141* (29), *mcl(3)Z4617* (5), *mcl(3)Z5121* (33), *mcl(3)Z5468* (32), *mcl(3)Z5502* (32), *mcl(3)Z5578* (30), *mcl(3)Z5839* (19), *mcl(3)Z5860* (28), *mcl(3)Z6257* (12)	This study; B. McKee (personal communication)
Postfertilization[c]	*paternal loss*	pal^{Z0483} (11)	This study
	loser	lsr^{Z1822} (24)	This study
ND[c]	ND	*mcl(2)Z0193* (22), *mcl(2)Z0756* (53), *mcl(2)Z2641* (7), *mcl(2)Z4635* (10), *mcl(2)Z5838* (10), *mcl(3)Z0375* (10), *mcl(3)Z0684* (15), *mcl(3)Z1335* (9), *mcl(3)Z1345* (23), *mcl(3)Z2566* (8), *mcl(3)Z2585* (5), *mcl(3)Z2592*, *mcl(3)Z3808*, *mcl(3)Z3946* (15), *mcl(3)Z6256* (20), *mcl(3)Z6259* (12)	This study

ND, not determined.

[a] All *mcl* lines gave fourth chromosome loss rates of ≥5% when scored in the initial assay; the percentages shown here are the average percentage of fourth chromosome loss based on three or more separate trials.

[b] Timing of chromosome loss as meiotic is based on the observation of micro- and/or macro-nuclei in onion-stage spermatids.

[c] For these lines, there was no cytological evidence for chromosome loss during spermatogenesis.

mentation group called *nuclear blebber* (*nbl*), led to the production of micronuclei in gonial cells and in young primary spermatocytes. Micronuclei were also detected at subsequent stages of spermatogenesis in the *nbl* males. The fragmentation of nuclei and prevalence of the micronuclei in mature primary spermatocytes were particularly striking (Figure 2). The size of the micronuclei indicated that the premeiotic chromosome loss induced by *nbl* was not restricted to the small fourth chromosome.

Characterization of five mutations from this collection by Tomkiel *et al.* (2001) identified *teflon*, a gene limited in its effects to the male germline and required for meiosis I segregation of the autosomes, but not the sex chromosomes. Other *mcl* genes were more general in action, affecting chromosome transmission in both sexes (R. S. Hawley, B. McKee, J. Tomkiel and B. T. Wakimoto, unpublished results). Complementation analyses showed that *Z5736* carries an allele of *ord* (B. McKee, personal communication), a gene required to maintain sister-chromatid cohesion during meiosis in males and females (Mason 1976; Goldstein 1980).

As shown in Table 3, the remaining *mcl* mutations were grouped according to cytological observations. We were unable to deduce the time of chromosome loss for 15 lines either because homozygotes were inviable by the time the assay was performed or because they showed apparently normal spermatogenesis by our cytological assay. The testis squashes we used were low resolution, so infrequent chromosome loss, particularly of the fourth chromosome, may have been missed. Thirty lines showed detectable cytological defects. A large number of *mcl* lines showed apparently normal primary spermatocyte nuclei but prevalent micronuclei, and sometimes also macronuclei, in onion-stage spermatids. These cytological features indicate that chromosome loss in these lines was minimally meiotic (although loss could continue to occur postmeiotically as well) and that the loss events were not restricted to the tiny fourth chromosome.

Two lines were unusual in that mutant males regularly produced mosaic progeny that had large patches of somatic tissue lacking the paternal fourth chromosome. These males also produced a significant number of nullosomic progeny, which may have resulted from chromosome loss during the early syncytial divisions of the embryo. *Z0483* carries a second-chromosome mutation that is an allele of *paternal loss* (*pal*). The original *pal*

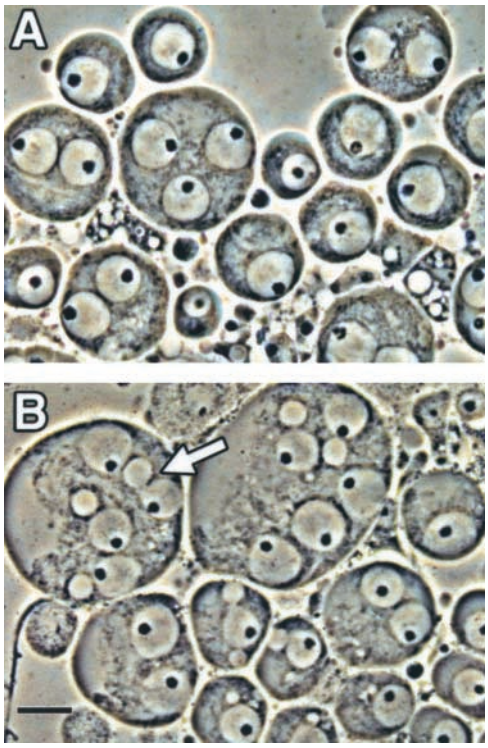

Figure 2.—The unusual mutant phenotype of *nuclear blebber*. (A) Growing primary spermatocytes from normal males. The large mature spermatocytes have uniformly sized nuclei (white) and prominent nucleoli (dark dots). The cytoplasm appears gray. The preparation has been flattened slightly and a few of the cytoplasmic bridges between the cells are broken. (B) Variable sizes of the primary spermatocyte nuclei of nbl^{Z1837} males and nuclear fragmentation by blebbing (arrow). Bar, 20 um.

mutation, pal^{1}, was previously classified as a meiotic mutation (Baker 1975). However, it has since been shown that *pal* is a strict paternal-effect gene (Owens 1996). *Z1822* carries a third-chromosome mutation that defines a new gene required for stable maintenance of the paternal chromosomes in the embryo. We have named this gene *loser* (*lsr*; S. Eacker and B. T. Wakimoto, unpublished results).

DISCUSSION

This article describes a screen for recessive male-sterile mutations of ~12,000 lines that carry in balanced condition second or third chromosomes treated with high doses of ethyl methanesulfonate. The screen yielded 2396 lines with recessive male sterility or barely fertile phenotypes and 62 lines with abnormal transmission of the paternal fourth chromosome. Although previous screens have been performed for male-sterile mutations of Drosophila using X rays, *P* elements, and EMS, with the largest of these yielding 400 EMS-induced male-sterile strains (Hackstein 1991), the Z *ms* collection is the largest collection of male-sterile mutations yet described for any organism. It therefore offers an unprecedented opportunity to understand the genetic regulation of male fertility. Additional advantages of this collection include the nonspecific nature of the mutagen and the homogeneous genetic background upon which the mutations were induced. Furthermore, as described by Koundakjian *et al.* (2004, accompanying article), the Z collection has been screened by numerous laboratories for mutations affecting a wide variety of biological functions other than male fertility; thus additional phenotypes associated with the these lines are being discovered.

Our screen is confined to second and third chromosomes, four-fifths of the Drosophila genome. The screen is incomplete in that the X and fourth chromosomes have not been examined. However, the fourth chromosome represents <1% of the genome, and published results suggest that the testis-expressed genes are dramatically underrepresented on the X chromosome (Joslyn 1988; Parisi *et al.* 2003). Genetic analyses of the *ms* and *mcl* mutations are well under way and mutations affecting specific aspects of spermatogenesis are being pursued in many laboratories throughout the world (see, *e.g.*, White-Cooper *et al.* 2000; Hiller *et al.* 2001; Tomkiel *et al.* 2001; Schultz *et al.* 2002; Tazuke *et al.* 2002; Bucciarelli *et al.* 2003; Farkas *et al.* 2003; Greene *et al.* 2003). The genetic studies reveal that, for some loci, many alleles are present in the collection, whereas others are represented by only one or a few alleles (*e.g.*, see Koundakjian *et al.* 2004 and Laurencon *et al.* 2004, accompanying articles in this issue). For the *ms* collection, low numbers of alleles at some loci possibly reflect the fraction of male-sterile alleles among all possible mutations in a locus and not the total mutation rate per locus (Koundakjian *et al.* 2004, accompanying article). For some loci, virtually all mutations are male sterile and, for others, only rare mutations lead to a nonlethal phenotype; for example, some male-sterile mutations can be shown to be alleles of essential genes in that they are not complemented by specific lethal mutations mapping to the same region (Lifschytz and Yakobovitz 1978; D. L. Lindsley and J. Roote, unpublished results).

Considerations discussed in the accompanying article by Koundakjian *et al.* (2004) provide an estimate of the coverage of the Z collection. They argue that the dose of EMS used was sufficient to induce in a sample of the size studied here 7–9 mutations in each gene, and they report that some genes are represented by as many as 15 Z mutations in the collection. The genetic analyses of the *ms* and *mcl* mutations described here show that coverage of the collection is sufficient to recover alleles in genes that had been relatively underrepresented in previous screens for male-sterile mutations (Hackstein 1991; Castrillon *et al.* 1993; Fitch *et al.* 1998). For instance, we recovered new Z alleles for all three paternal-effect genes that had been known to date

(*pal, K81*, and *snky*) and have identified mutations in six new paternal-effect genes (K. Fitch, K. Wilson and B. T. Wakimoto, unpublished results), one of which has 11 alleles. In addition, in the process of screening the second-chromosome collection, we recovered nine lines that failed to produce males and we have subsequently verified that these are distributed as 3 alleles each of the known male-specific lethal genes, *msl-1*, *msl-2*, and *mle* (see materials and methods).

The size of the male fertility genome in Drosophila may be estimated using genetic or molecular data. Genetic data, including those reported here, can provide information on the number of genes mutable to male sterility while expression data can provide a measure of the proportion of the genome expressed preferentially in males or in the testis. Both approaches are imperfect for estimating the number of male fertility genes because of certain underlying assumptions. For instance, in our screen, the criterion for male sterility was stringent so only a few mutations that lead to significant reduction but not absolute sterility (those we called "barely fertile") were not included in the *ms* collection. In addition, genes that mutate only rarely to male sterility will be underrepresented in genetic screens. These factors limit the use of our data to an estimate of the minimum number of male fertility genes. On the other hand, molecular screens will most certainly overestimate the gene number since transcripts enriched in males or in the testis will not necessarily be required for male fertility.

With the above caveats in mind, we attempt an estimate of the number of male fertility genes. The in-depth knowledge of a particular region of chromosome 2 (the Adh region) allows us to use our data to provide a rough estimate of the minimum number of genes that can mutate to male sterility. All of the second-chromosome male-sterile lines were crossed to a series of deficiencies in the Adh region; 51 fell into the segment tested, and complementation tests identify 13 complementation groups (D. L. Lindsley, unpublished results). Extrapolating to 962 mutations (Table 1) implies 245 complementation groups on chromosome 2 that can mutate to male infertility with an average of about 4 alleles per gene (51/13). This approach to estimating male fertility genes is independent of the assumptions of Poisson distributions, but does assume that the Adh region is representative of the major autosomes. This number may not reflect the total number of genes required for male fertility; for example, males carrying temperature-sensitive sex-linked lethal mutations, when reared under permissive conditions, are male sterile or become so when held at restrictive temperatures in 10% (Shellenbarger and Cross 1979) to 40% (Lifschytz and Yakobovitz 1978) of cases tested.

As mentioned previously, genes showing male-biased expression are underrepresented on the X chromosome (Joslyn 1988; Parisi *et al.* 2003). Joslyn (1988) isolated a number of testis-expressed cDNAs and mapped them cytologically by *in situ* hybridization to polytene chromosomes. Among 154 cDNAs that were expressed in the testis, but not in somatic tissues, 9 mapped to the X chromosome and 75 to the second chromosome. Applying this ratio to the 245 second-chromosome complementation groups estimated above yields an estimate of 27 loci on the X that can mutate to male sterility. Parisi *et al.* (2003) report a highly significant ($P < 0.0001$) underrepresentation of genes with male- or testis-biased expression on the X chromosome compared to the autosomes that is based on microarray analyses of over 14,000 predicted transcripts. Finally, assuming chromosomes 2 and 3 to be approximately equally mutable, we arrive at an estimate of a minimum of 500 male fertility genes in the genome.

The estimate of 245 such genes on chromosome 2 and 1247 essential genes (Koundakjian *et al.* 2004, accompanying article), a significant fraction of which may function in the testis (Lifschytz and Yakobovitz 1978; Shellenbarger and Cross 1979), implies that male-sterile mutations are ~20% as frequent as lethal mutations. Considering only those lines designated as fully sterile, we can estimate from the incidence of nonsterile lines that there were 0.20 male-sterile mutations per second chromosome and 0.22 per third chromosome. Comparing these mutation rates with those for essential genes estimated from the same screen (Koundakjian *et al.* 2004, accompanying article), we can estimate that mutation rates for male sterility are 10.8 and 13.0%, respectively, of those for essential genes. These two independent estimates of 20% and 10.8–13% bracket an earlier estimate of 15% made by Lindsley and Lifschytz (1972).

The classification of the *ms* lines into phenotypic categories provides a rough idea of the relative proportions of genes affecting different stages of spermatogenesis. Relatively few of the mutant lines (8% of the collection) affect premeiotic events, such as specification and proliferation of the germline, the transition from gonial to primary spermatocytes, or entry into meiosis, while 20% of the collection showed abnormalities in meiosis, and 53% lacked gross defects at early stages, but affected spermiogenesis. The fact that the "classic" male-sterile class represented over half of the collection and 68% of those showing defects in spermatogenesis reflects the high sensitivity of the final stages of spermatid differentiation to genetic disruption. The failure to individualize sperm may be the manifestation of earlier defects, with the process of individualization acting as an efficient checkpoint to eliminate abnormalities from the sperm pool (Tokuyasu *et al.* 1977).

To date, most studies of male-sterile mutations have focused on those that are required for spermatogenesis. Our results showed that these constitute the majority (81%) of mutations that affect male fertility. The remaining 19% have motile sperm and affect postsperma-

togenic processes. Although these mutants have been recovered in the past and constitute a sizable fraction of *ms* mutations, they are relatively understudied. Further categorization of the >400 mutations in this class is in progress and shows that this is a highly heterogeneous group in terms of mechanisms of action. Included in this group will be mutations that affect the behavioral, anatomical, and molecular requirements for mating and sperm function, including transfer to the female, storage in the sperm storage organs, and fertilization competence.

A major goal of our screen was to identify genes required for stable inheritance of chromosomes through the male germline and in the early embryo. Preliminary characterization of the *mcl* lines showed that we recovered cases of premeiotic, meiotic, and postfertilization fourth chromosome loss, with a variety of underlying mechanisms represented. Those exhibiting meiotic loss were the most common. We anticipate that many of these are hypomorphic alleles of the *ms* mutations that showed variably sized spermatid nuclei and resulted in complete male sterility. An allele of *ord* that affects female meiosis was recovered in our screen (B. McKee, personal communication). This mutation and other *mcl* lines with strong effects in females should be encountered in ongoing screens of the Z collection for female meiotic mutants (Giunta *et al.* 2002; R. S. Hawley, K. McKim and J. Sekelsky, personal communication). Comparisons of the results of these screens with our data will provide information on the relative numbers of mutations affecting meiosis in both sexes or that are specific to either sex. In *D. melanogaster*, male meiosis differs from female meiosis in lacking synaptonemal complexes, meiotic recombination, and distributive disjunction of nonexchange chromosomes. Males also have special requirements for X-Y pairing (McKee 1998) and show a striking compartmentalization of bivalents during prophase (Vasquez *et al.* 2002). The size of the *mcl* collection predicts that many new genes specifically affecting chromosome pairing or achiasmate segregation in males are likely represented by more than one allele in the collection. Tomkiel *et al.* (2001) showed that one such gene, called *teflon*, is represented by five Z alleles. Interestingly, male meiotic mutations exhibit chromosome specificity more commonly than do female meiotic mutations (Ivy 1981) and *teflon* specifically affects the autosomes. The *mcl* collection will be useful for identifying genes required for transmission of all chromosomes, just the autosomes, or specifically the fourth chromosome. Our screen was limited in that we did not recover mutations that specifically affected transmission of the sex chromosomes. In theory, we could have identified such mutations if they had also induced high levels of paternal X chromosome loss in the embryo; however, in practice, these events were not detected in our screen.

As the Zuker *ms* and *mcl* collections are further characterized genetically, allelic relations as well as genetic and cytological positions of many more Z mutations will become defined. By bringing to bear genomic sequence and testis-specific cDNA information (Andrews *et al.* 2000), the molecular identification of nearly all of the genes that contribute to male fertility in Drosophila is possible. Comparisons of these data with the information on male-sterile mutations and male-enriched transcripts of other organisms, including *Caenorhabditis elegans* (L'Hernault 1997; Jiang *et al.* 2001), the mouse, and humans (Matzuk and Lamb 2002; Ostermeier *et al.* 2002), will allow for a more comprehensive understanding of the evolutionarily conserved genetic basis of male fertility.

We are deeply indebted to Charles Zuker for allowing us to screen the Zuker collection, for generously providing space and support for all aspects of this project, and for making the lines available to the Drosophila research community. We are grateful to Edmund Koundakjian for his dedication in generating and expertly maintaining the Z collection, and we thank him and Maureen Cahill for providing stocks for this study. B.T.W. also thanks Robert Hardy for his advice and encouragement during the critical beginnings of this project and Larry Goldstein for hosting her sabbatical visit at the University of California at San Diego. We acknowledge our colleagues for allowing us to cite their unpublished data as noted in the text and Table 3 and members of the Wakimoto lab for discussions and comments on the manuscript. This work was supported in part by a grant from the National Eye Institute of the National Institutes of Health to Charles Zuker and by National Science Foundation grants (HRD962704 and DCB0211733) to B.T.W.

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Communicating editor: T. C. Kaufman