Toward a Molecular Genetic Analysis of Spermatogenesis in *Drosophila melanogaster*: Characterization of Male-Sterile Mutants Generated by Single *P* Element Mutagenesis

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ABSTRACT

We describe 83 recessive autosomal male-sterile mutations, generated by single *P* element mutagenesis in *Drosophila melanogaster*. Each mutation has been localized to a lettered subdivision of the polytene map. Reversion analyses, as well as complementation tests using available chromosomal deficiencies, indicate that the insertions are responsible for the mutant phenotypes. These mutations represent 63 complementation groups, 58 of which are required for spermatogenesis. Phenotypes of the spermatogenesis mutants were analyzed by light microscopy. Mutations in 12 loci affect germline proliferation, spermatocyte growth, or meiosis. Mutations in 46 other loci disrupt differentiation and maturation of spermatids into motile sperm. This collection of male-sterile mutants provides the basis for a molecular genetic analysis of spermatogenesis.

SPERMATOGENESIS in Drosophila begins with the formation of a spermatogonium from a population of germline stem cells. A fixed number of mitotic division ensues, followed successively by cellular growth, meiosis, and an elaborate pathway of cellular differentiation. Throughout this process the developing germ cells are accompanied by a pair of somatic cells. The entire program from stem cell division to functional sperm requires 10 days, and is initiated every 10 hr. Thus, all stages of spermatogenesis are present in a single adult testis, with earlier stages at the tip and later ones at the base (for reviews see LINDSLEY and TOKAYASU 1980; FULLER 1993).

The opportunity to explore the maintenance of stem cell identity, the regulation of an invariant number of mitotic divisions, the execution of meiosis, the initiation of a program of morphogenesis, and the interaction between germ line and soma make spermatogenesis an ideal target for investigations into differentiation and cell-cell interaction.

Despite a detailed understanding of spermatogenesis at the morphological level, relatively little is known about the genes controlling this differentiation program. Exceptions include the testis-specific β_2-tubulin and the *cdc25* homolog *twine*, both of which were identified as homologs of gene products studied in other organisms (KEMPHUES *et al.* 1979; ALPHEY *et al.* 1992; COURTOT *et al.* 1992). Male-sterile mutants defective for different stages of spermatogenesis have been generated by chemical mutagenesis in screens conducted by several laboratories (see for example LIFSCHYTZ 1987; HACKSTEIN *et al.* 1990; HACKSTEIN 1991). However, most of the mutants generated in this manner have not been mapped, and the majority are no longer available.

We have chosen a different approach, that of single *P* element insertional mutagenesis (COOLEY, KELLEY and SPRADLING 1988), to conduct a genetic analysis of spermatogenesis in *Drosophila melanogaster*. With this method, each mutation is genetically tagged by an eye color marker and can be readily located on the physical map by *in situ* hybridization to polytene chromosomes. Furthermore, new mutations, including null alleles, can be efficiently generated by remobilization of the *P* element with a transposase source. Lastly, DNA flanking an insertion site can be readily cloned by bacterial transformation or by polymerase chain reaction (PCR), greatly facilitating molecular analysis of the locus.

We present here the isolation and phenotypic characterization of 83 male-sterile autosomal mutations generated by single *P* element mutagenesis. The vast majority affect spermatogenesis, with defects ranging from nonproliferation of germ cells to the production of defective sperm. The results of complementation tests with available chromosomal deficiencies, together with reversion analyses, indicate that the *P* element insertions are responsible for the observed male sterile phenotypes. These mutations should therefore provide a starting point for the dissection

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of mechanisms controlling the course of spermatogenesis.

MATERIALS AND METHODS

Genetic strains: All marker mutations and balancer chromosomes are described and referenced by LINDSLEY and ZIMM (1992). Crosses were carried out at 25° in vials containing freshly yeasted cornmeal molasses agar. Oregon R, Sevelen (EDGAR and SCHUBIGER 1986), *st e* or w^{1118} flies were used as wild type.

Generation of single *P* element insertion lines: The *P[lacZ, ry⁺]* element, hereafter called *P[Z]*, contains a drug resistance gene, plasmid replication sequences, the Drosophila *rosy*⁺ gene, and a fusion of the *lacZ* gene to the first exon of the *P* transposase gene (MLODZIK and HIROMI 1992). The crossing scheme used for generation of insertions of the *P[Z]* transposon is described elsewhere (KARPEN and SPRADLING 1992). In brief, a *P[Z]* element on the *X* chromosome was mobilized by introducing the Δ2-3 *P* transposase source (ROBERTSON *et al.* 1988). Approximately 8000 lines were generated containing new insertions of *P[Z]* in an isogenized *cn* second chromosome or a ry^{506} third chromosome. Mutations were balanced with *CyO* for second chromosome insertions and with one of four chromosomes (*MKRS;TM3, ry;TM6B, ry;* or *CxD*) for third chromosome insertions.

An additional set of single *P* element insertion lines carrying *P[lac, ry⁺]A* (O'KANE and GEHRING 1987) was similarly generated by using the Δ2-3 transposase source and was generously provided by the UCLA fly group. Insertions of this transposable element were on unmarked second chromosomes or third chromosomes carrying ry^{506}.

Identification of males-sterile mutants: Male sterile mutants were identified among the single *P* element lines by performing fertility assays: Eight to ten homozygous males were crossed with an equal or greater number of virgin wild-type females. Homozygotes were designated as sterile if there were no progeny from these crosses, and as semisterile if they produced fewer than 10% the number of progeny produced by heterozygous siblings.

Viability for each male sterile line was measured among the progeny of a cross between balanced heterozygotes. Mutations were deemed to be semilethal if homozygotes were present at less than 30% the expected frequency.

Female fertility was assayed by crossing at least five homozygous females from a given line with an equal number of balancer sibling males.

***In situ* hybridization:** Larvae for salivary gland squashes were generated by outcrossing each mutant line to wild type. After 2–3 days at 25°, the vials were transferred to 18° and supplemented with fresh yeast. *In situ* hybridizations and Giemsa staining were carried out essentially as described (ASHBURNER 1989). The *P[Z]*-containing plasmid pHZ50PL (a gift of Y. HIROMI), the *P[lArB]* plasmid (WILSON *et al.* 1989), or genomic DNA flanking the insertion site was used as probe.

Complementation and deficiency crosses: Complementation tests were performed by crossing each male-sterile mutation from the collection to chromosomal deficiencies potentially spanning the insertion site, as well as mutations that mapped within two lettered polytene divisions, including: 1) other insertion mutations from the collection; 2) other cytologically localized, male-sterile mutations induced with *P* elements (COOLEY, BERG and SPRADLING 1988); 3) alleles of *β2t* and *fru* (KEMPHUES *et al.* 1979; GAILEY and HALL 1989); and 4) mutations that are both male- and female-sterile from the collection of SCHÜPBACH and WIESCHAUS (1991).

Reversion analysis: Insertion-bearing chromosomes were brought together with a transposase source by crossing flies from the balanced mutant stocks to flies carrying the *P[ry⁺ Δ 2-3]* transposase source on either the second or third chromosome (ROBERTSON *et al.* 1988; M. SANICOLA and W. GELBART, personal communication). In the next generation, the *P[ry⁺ Δ 2-3]* chromosome was crossed out and *ry* derivatives of the *P[Z]* or *P[lac, ry⁺]A* chromosome were selected and used to establish lines. The *ry* derivatives were then made homozygous or put in *trans* to the original insertion for tests of male and female fertility.

Characterization of male-sterile phenotypes: For gross examination of reproductive organs, five males, one to seven days old, homozygous for the *P* element insertion were dissected in modified Drosophila Ringer's solution (5 mM PIPES, pH 6.9; 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$), Becker's Ringer's solution or phosphate-buffered saline (PBS) (ASHBURNER 1989). Seminal vesicles were torn open with forceps under a dissection microscope and examined for the presence and motility of sperm. Testes from mutants that failed to produce motile sperm were analyzed by phase contrast microscopy and by X-gal and Hoechst staining, as described below. For those male-sterile mutants that produced motile sperm, transfer of sperm to females was assayed by dissection of the sperm storage organs of females with whom they had been mated. Mutants that failed to transfer their motile sperm to females were further analyzed for behavioral or anatomical defects.

Examination of testis contents by phase-contrast microscopy: For the examination of testis contents by phase contrast microscopy, testes from at least four newly eclosed males were dissected out with forceps and placed on a glass slide with a drop of Ringer's solution or PBS. The testes were torn open and gently squashed under the weight of a cover slip (KEMPHUES *et al.* 1980). Spermatogenic cells of stages before, during and shortly after meiosis were visualized by this procedure.

X-gal and Hoechst staining of testes: To determine the pattern of expression of the enhancer-trapping *lacZ* gene in the *P* element, testes from newly eclosed adults were stained with X-gal as described (GÖNCZY, VISWANATHAN and DINARDO 1992). To assess the shape and distribution of germ cell nuclei, testes from newly eclosed males were fixed with 4% formaldehyde (EM grade, Polyscience) for 20 min in PBX (0.1% Triton X-100 in PBS), washed three times for 5 min in PBX, stained with 1 μg/ml Hoechst 33258 (Sigma) for 2 min in PBX, rinsed in PBX, washed for 30 min in PBS and mounted in Fluoromount-G (Fisher).

Classification of male-sterile mutations: Male-sterile mutants were first categorized with regard to whether they produced normal amounts of motile sperm in the seminal vesicle or were defective for spermatogenesis. The mutants were then grouped into seven general classes, five for those affected in spermatogenesis and two for those producing wild-type levels of motile sperm. Classification was based on the earliest stage at which visible defects were readily and reproducibly apparent in homozygotes.

Proliferation phase defect: A reduction or absence of growth phase spermatocytes is apparent at eclosion or a few days post-eclosion. Such testes are typically thin and in more extreme examples resemble those of agametic mutants.

Growth phase defect: Germ cells complete the proliferation stage and form cysts of 16 early spermatocytes, but undergo aberrant development during the primary spermatocyte growth phase.

Meiotic entry defect: Spermatogenesis is normal through the late spermatocyte stage, but meiosis does not occur.

Meiotic division defect: Meiosis is initiated but fails to give rise to the normal pattern of 64 spermatids.

Postmeiotic differentiation defect: Mutants undergo normal meiosis, but fail to produce mature sperm. The mutants which fall into this category are readily distinguishable from wild-type by the absence of sperm in the seminal vesicle.

Behavioral defect: Homozygous males produce motile sperm at wild-type levels, but fail to copulate with virgin females during 30-min observation periods (GAILEY and HALL 1989).

Sperm transfer defect: Motile sperm are found in the seminal vesicle at wild-type levels, but no sperm transfer occurs. In one mutant, mating is observed but few or no sperm are found in the female storage organs. In other mutants, the external genitalia of homozygous males are aberrant or absent.

Naming of loci: The results of genetic tests governed the naming of loci: 1) in cases where our mutations were found to be alleles of previously characterized loci, the preexisting name, if any, was adopted; 2) in cases where more than one allele was identified in our screen, or where alleles were identified among available male-sterile mutations of unnamed loci, we assigned the locus a name according to our phenotypic characterization; 3) we also gave names to any of the loci for which we isolated transposase-induced revertants or identified deficiencies that failed to complement the male-sterile phenotype; 4) all other single allele loci were given designations based solely on cytological location.

RESULTS

Our collection of male-sterile mutations was gathered in two stages. First, a screen for male-sterile mutants was carried out such that the frequencies of different classes of mutants (lethal, female-sterile, male-sterile, and male- and female-sterile) could be tabulated (Table 1). This set was then supplemented with mutants drawn from additional screens. Mapping, complementation tests, and phenotypic analyses were performed on the combined collection. We begin by presenting an overview of the results from the first mutant screen. We then consider the complete collection and describe each of the phenotypic classes in turn. Representative mutants are highlighted in the text and figures.

Isolation of male-sterile insertion mutations: From approximately 8000 autosomal *P[Z]* element insertion lines, we screened a subset of 1919 for male sterility with no preselection other than for some degree of homozygous viability (Table 1A). Twenty-eight (1.5%) caused partial or complete male sterility; 4 of these 28 also affected female fertility. An additional 48 inserts (2.5%) were strictly female-sterile. Viability was good (>70% expected) for 26 of the 28 male-sterile lines. Twenty-four mutations affected spermatogenesis; the remaining 4 led to anatomical or behavioral defects. Of the spermatogenesis mutants, the majority affected postmeiotic differentiation; only 5 of the 24 disrupted earlier stages (Table 1B).

Of the 28 events affecting male fertility, 15 mapped to the second chromosome and 13 to the third. Southern analysis using a ~0.5-kb probe from the 5′ end of the *P* element revealed a single band in genomic digests from each of the 28 lines (data not shown). Thus each male-sterile line from this set contains a single *P* element.

To confirm that the *P* element was responsible for the mutant phenotype, we used a transposase source to promote excision of the *P[Z]* element from seven different insertion lines. For all seven lines, two or more independent *ry* derivatives were male fertile when homozygous or in *trans* to the original insertion. Moreover, the parental DNA restriction map at the site of insertion was restored for the subset of five lines which were analyzed on Southern blots (data not shown).

To further test whether the single *P* element insertion was responsible for the recessive male-sterile phenotype, we asked whether sterility was observed when an insertion was placed in *trans* to a cytologically defined deficiency spanning the insertion site. Such deficiencies were available for six of the seven lines for which we generated revertants, as well as for six others from among the 28 lines. In all 12 cases, the insertions were male-sterile in *trans* to the deficiency. In several instances, the insertion in *trans* to a deficiency also showed female sterility or decreased viability. These results will be discussed in more detail below.

Analysis of an expanded collection of male-sterile mutants: In order to study the broadest possible array

TABLE 1

Screen for male sterile mutations among a set of random single *P* element insertions on the second and third chromosomes

	n	% Total hits
A. Total insert lines	1919	100
Homozygous lethal insertions	262	14
Female sterile (male fertile)[a]	48	2.5
Male sterile (female fertile)[a]	24	1.3
Male and female sterile[a]	4	0.2
B. Phenotypes of male sterile insertions[a,b]	***n***	**% Male Steriles**
I. Defects in spermatogenesis		
Proliferation defect	2	7
Growth phase defect	1	4
Meiotic entry defect	0	—
Meiotic division defect	2	7
Postmeiotic differentiation defect	19	68
	24	
II. Defects in mating		
Anatomical	1	4
Behavioral	3	11
	4	

[a] Sterile classes include a few semisterile lines which produce a limited number of progeny, no more than 10% the number seen with wild-type flies (see Table 2).

[b] Includes all male-sterile lines, both female-fertile and female-sterile.

of phenotypes and to obtain additional alleles of loci of interest, we identified more male-sterile mutations among the remaining *P[Z]* lines, as well as an additional set of single element insertions generated with *P[lac, ry$^+$]A* (see MATERIALS AND METHODS). Since the additional lines examined did not include all viable mutants generated, we did not measure the frequency of male-sterile mutations in these cases. In all, we identified 69 *P[Z]*-induced mutants as well as 14 induced with *P[lac, ry$^+$]A*. *In situ* hybridization with a transposon-derived probe revealed a single insertion in each of these lines (Table 2). Six male-sterile lines for which *in situ* hybridization detected no element or more than one *P* element were not included in the collection.

Mapping and complementation experiments indicated that the 83 male-sterile or semisterile mutations affect 63 loci on the second and third chromosomes. For about three-fourths of these mutations (61/83), we have used excision of the *P* element or complementation tests with deficiencies or other mapped insertion alleles to confirm that the insertion is responsible for the mutant phenotype (Table 3).

The mutants have been placed into seven phenotypic classes, five of which involve defects in spermatogenesis and two of which affect mating or sperm transfer.

Proliferation phase defects: Mutations in two loci cause early defects in spermatogenesis that result in testes with reduced germinal content. In wild-type testes, the various stages of spermatogenesis are ordered from the tip to the base of the testis. Individual spermatogonia arise by asymmetric divisions of germline stem cells at the tip of each testis (Figure 1a). Each spermatogonium becomes surrounded by a pair of cyst cells derived from a distinct, somatic stem cell population (Figure 1b). The spermatogonium and the enveloping cyst cells comprise a cyst which progresses as a unit through spermatogenesis. Within each cyst, the spermatogonium undergoes four mitotic divisions, generating 16 spermatocytes, whereas the two cyst cells do not divide (Figure 1c). In mutants affecting the proliferation phase, a defect in the early stages of spermatogenesis results in the underrepresentation or absence of subsequent stages, and the testes are thin and short.

The phenotype of males mutant for *diaphanous* (*dia*) at 38E is unusual in exhibiting a dramatic dependence on the age of the fly. At eclosion, testes of *dia* flies are somewhat thinner and shorter than wild-type (compare Figure 2, a and b). As a *dia* male ages, the early stages of spermatogenesis are not replenished, resulting in very thin testes, devoid of germinal contents (Figure 2c). The *dia* mutation is thus likely to affect the proliferation stage of spermatogenesis. In addition, defects in meiosis are observed in testes from young *dia* males. These results suggest that *diaphanous* is required in males for both mitotic and meiotic germ cell divisions. In *trans* to a deficiency, *dia* males have a similar phenotype but females become semisterile, producing ovaries with few egg chambers. We infer that *dia* function is also required for germ cell divisions in females.

Proliferation phase defects are also apparent in males homozygous for mutations in the *chickadee* (*chic*) locus at 26A. *chic* testes are reduced in size to a variable extent; an example of a mildly affected testis is shown in Figure 2d. The phenotype becomes more severe over a deficiency. The testes of *chic^{11}/Df* males are short and thin (Figure 2e) and nearly devoid of germinal content. They thus resemble the agametic testes of germlineless progeny of *oskar* mothers (Figure 2f). Some of our *chic* alleles affect female fertility. All previously isolated alleles of the *chic* locus are female-sterile; some are also male-sterile (SCHÜPBACH and WIESCHAUS 1991; COOLEY, VERHEYEN and AYERS 1992).

Comparison of the testis phenotypes of *osk*, *chic* and *dia* illustrates a relationship between the size of the testis and the extent of germline division and growth. When the germline is absent *ab initio*, as in the progeny of *osk/osk* mothers, the testis is severely stunted (Figure 2f). When germline cells are present in larval, but not adult, gonads, as in the case of *chic/Df* (data not shown), the adult testes is somewhat larger than a germlineless testis (compare Figure 2, e and f). Last, when germ cells are present in the gonads of both larvae and young adults but are not replenished, as in *dia* males at day 5, the testis is larger still (Figure 2c).

Growth phase defects: Mutations in five loci result in defects first detectable during the spermatocyte growth phase that follows the proliferative period. In the wild-type growth phase, high levels of transcription accompany a 25-fold increase in spermatocyte volume (Figure 1, c and d). Mature spermatocytes have large nuclei with prominent nucleoli (Figure 3a). Spermatocytes from growth phase mutants are altered in appearance, exhibiting defects in the morphology of nuclei or other cellular structures.

The *bocce* (*boc*) mutation at 51D results in spermatocyte cysts that contain nuclei of variable size (Figure 3b). The number of nuclei per cyst is also somewhat variable, but is on average 16. In addition, there is an apparent absence of cell boundaries within a cyst. This can be observed both in spermatocyte cysts (Figure 3b), as well as in rare meiotic cysts, in which mitochondria that normally segregate to each of the haploid cells are found fused into one or two giant masses (Figure 3c, arrow; compare with Figure 5a). The absence of cell boundaries raises the possibility that *bocce* is required for proper cytokinesis during the gonial divisions.

TABLE 2

Loci on second and third chromosomes mutating to male sterility

Locus/Cytology	Allele numbers	Phenotypic description[a]
Proliferation defect (2 genes)		
chickadee/26A	*chic*^{10}, *chic*$^{11(b)}$, *chic*^{12}, *chic*^{13}, *chic*^{14}, *chic*^{15}	Testes have reduced germinal content. Alleles *chic*^{10} and *chic*^{14} are male semisterile. Allele *chic*^{13} is female semisterile. Allele *chic*^{11} is female sterile. In *trans* to a deficiency, inserts are male- and female-sterile and phenotypes are more severe. Locus was discovered by SCHÜPBACH and WIESCHAUS (1991). Encodes profilin (COOLEY, VERHEYEN and AYERS 1992).
diaphanous/38E	*dia*^{1}	Late-stage cysts are present at eclosion. Testes are empty in 5-day-old males. Insert is male sterile and female semisterile in *trans* to deficiency. Ovaries contain few egg chambers. Null alleles are lethal (D. CASTRILLON and S. WASSERMAN, unpublished results).
Growth phase defect (5 genes)		
bocce/51D	*boc*^{1}	Nuclear size and number is variable in spermatocyte cysts. Infrequent spermatid cysts are highly aberrant. Testes are small. Females are semisterile; few eggs are laid. Phenotype is more severe in *trans* to deficiency: no spermatids are found in males; females lay no eggs.
cueball/62A	*cue*^{2}	Spermatocytes contain cytoplasmic abnormalities. Rare post-meiotic cysts are defective. Testis is short with defective sheath. Females are semisterile; ovaries are small and misshapen. Phenotypes are more severe in *trans* to a deficiency. Locus was discovered by P. G. WILSON and M. T. FULLER (personal communication).
fumble/77B	*fbl*^{1}, *fbl*^{2}	Testes from *fbl/fbl* males contain spermatids with large nebenkerne and micronuclei at onion stage. Testes from *fbl/Df* males contain degenerating spermatocytes. Classification is tentative. Phenotype is more severe in *trans* to deficiency and earlier spermatogenic stages are affected; testes are short and nearly devoid of germinal content.
scratch/63AB	*stc*^{1}	Needle-shaped crystals accumulate throughout developing germline. Spermatids contain nuclei and nebenkerne of variable size. Phenotype resembles that of *XO* males.
l(2)26Ab/26A	*l(2)26Ab*$^{4(b)}$, *l(2)26Ab*^{5}	Testes are short and filled with cysts of 16-cell spermatocytes, which degenerate prior to completion of the growth phase. Mutation is allelic to *l(2)gdh-2* (KOTARSKI, PICKERT and MACINTYRE (1983).
Meiotic entry defect (2 genes)		
boule/66F	*bol*^{1}	Some 16-cell cysts resemble those seen in *pelota* (see below); others contain multiple nuclei in addition to the abnormally large nebenkerne.
pelota/30C	*pelo*^{1}	Meiosis does not occur, but abnormally large nebenkerne form in late spermatocytes. Mutation is cold-sensitive, female-sterile, and semilethal.
Meiotic division defect (3 genes)		
bobble/82D	*bob*^{1}	A few spermatids per cyst have nuclei or nebenkern of abnormal size. Mutation is semilethal.
doublefault/32A	*dbf*^{1}	Size varies for both nuclei and nebenkern at onion stage. Spermatid nuclei fail to change shape.
shank/82C	*shk*^{1}	Spermatids contain two or four nuclei associated with a single large nebenkern. Females are semisterile. Phenotype is less severe in *trans* to deficiencies.
Postmeiotic differentiation defect (46 genes)		
arrest/33CD	*aret*^{9}	Mutation is semilethal in *trans* to deficiency, but male-sterile phenotype is no more severe. Locus was previously identified as male- and female-sterile (SCHÜPBACH and WIESCHAUS, 1991).
bellwether/58F	*blw*^{1}	Mutation is allelic to *ms(2)Pry58F* (COOLEY, BERG and SPRADLING 1988).
betel/78D	*bet*^{1}	
blanks/36AB	*bln*^{1}	
capon/85A	*cap*^{1}	Mutation is allelic to *ms(3)Pneo85A* (COOLEY, KELLEY and SPRADLING 1988).
cashews/88B	*cas*$^{1(b)}$	
dispersed/46C	*disd*$^{1(b)}$	Elongated spermatid nuclei are dispersed. Males are semisterile.
effete/88D	*eff*$^{6(b)}$, *eff*$^{7(b,c)}$, *eff*^{8}, *eff*$^{9(c)}$, *eff*^{10}, *eff*^{11}, *eff*^{12}, *eff*^{13}	Mutations are allelic to *ms(3)88D* (BERG and SPRADLING 1991)[c].

(*continued on p. 494*)

TABLE 2—*Continued*

Loci on second and third chromosomes mutating to male sterility

Locus/Cytology	Allele numbers	Phenotypic description[a]
emmenthal/56E	emm^{1}	Nebenkerne are vacuolated.
gelded/28D	gel^{1}	
gorp/83B	$gor^{1(b)}$	Males are semisterile.
goulash/97EF	$goul^{1(b)}$	
gruyère/98D	$gru^{1(b)}$	Nebenkerne are vacuolated. Mutation is female-sterile and semilethal.
halley/55A	$hal^{1(b)}$	Spermatid nuclei fail to elongate; mutation is lethal over deficiency.
hephaestus/100E	$heph^{1}$, $heph^{2}$	Tip of testis is dilated to approximately twice wild-type circumference.
jaguar/95F	jar^{1}, jar^{2}	Males are semisterile.
mulet/46F	$mlt^{1(b)}$	Mutation is allelic to *ms*(2)*ry-3* (BERG and SPRADLING 1991).
oxen/49C	ox^{1}	
peanuts/50D	$pea^{1(b)}$	
pistachio/68C	$pto^{1(b)}$	Males are semisterile.
purity of essence/28E	poe^{1}, poe^{2}	
Rb97D	$Rb97D^{1}$	Mutation is insertion in RNA-binding protein gene (KARSCH-MIZRACHI and HAYNES 1993)
seedless/84F	sdl^{1}, sdl^{2}	
scattered/30B	$scat^{1}$	Elongated spermatid nuclei are dispersed.
thousand points of light/86E	tho^{1}	Elongated spermatid nuclei are dispersed.
trail mix/57E	tmx^{1}	Males are semisterile.
ms(2)*21D*		
ms(2)*27C*		
ms(2)*27CD*		Mutation is semilethal.
ms(2)*29F*		
ms(2)*30C*		Males are semisterile; elongated spermatid nuclei are dispersed.
ms(2)*42A*		Spermatid nuclei are not clustered.
ms(2)*42D*		Males are semisterile.
ms(2)*43C*		Spermatids occasionally contain variably sized nuclei and nebenkern.
ms(2)*46BC*		Mutation is semilethal and female-semisterile.
ms(2)*46C*		Elongated spermatid nuclei are dispersed.
ms(3)*61CD*		
ms(3)*65E*		
ms(3)*72D*		Postmeiotic nuclei have sickled appearance.
ms(3)*73D*		
ms(3)*80*		
ms(3)*85D*		
ms(3)*89B*		
ms(3)*90E*		Mutation is semilethal.
ms(3)*98B*		Males are semisterile.
ms(3)*100EF*		Males are semisterile.
Behavioral defect (2 genes)		
cuckold/28A	cuc^{1}, cuc^{2}	Males are semisterile due to failure to court and mate females. Longevity of both males and females is decreased.
fruitless/91B	fru^{3}, fru^{4}	Males court females and males, but fail to mate. Male-specific abdominal muscle is reduced. Locus was previously described by GILL (1963) and by GAILEY and HALL (1989).
Sperm transfer defect (3 genes)		
ken and barbie/60A	ken^{1}	External genital structures are absent in some males and females. Aristae are sparse and unpigmented. Mutation is semilethal and both male- and female-semisterile.
pointless/61B	$ptl^{1(b)}$	Males are semisterile, with wild-type levels of motile sperm in seminal vesicle. Little or no sperm is transferred to females.
twig/89E	$twig^{1}$	Anal-genital plate is twisted in both sexes. Females are sterile.

[a] Mutations are male-sterile, female-fertile, and have good viability, unless otherwise noted. Phenotype of mutations over deficiency, where available, is similar unless noted.

[b] Mutation was induced with the *P*[*lac*, *ry*+]*A* element. All other mutations were induced with the *P*[*Z*] element.

[c] Alleles eff^{7} and eff^{9}, as well as two preexisting *P* insertions in *effete*, affect viability and female fertility, but heteroallelic combinations are viable and female-fertile.

TABLE 3

Male sterile loci by cytology

Polytene location	Locus name	Deficiency for locus	Deficiency breakpoints		R[a]
			Left	Right	
21D	*ms(2)21D*				
26A	*chickadee*	*Df(2L)GpdhA*	25D7-E1	26A8-9	√
26A	*l(2)26Ab*	*Df(2L)GpdhA*	25D7-E1	26A8-9	√
27C	*ms(2)27C*				
27CD	*ms(2)27CD*				
28A	*cuckold*				
28D	*gelded*				√
28E	*purity of essence*				
29F	*ms(2)29F*				
30B	*scattered*	*Df(2L)30A; C*	30A	30C	
30C	*pelota*	*Df(2L)30A; C*	30A	30C	√
30C	*ms(2)30C*				
32A	*doublefault*	*Df(2L)J2*	31B	32A	
33CD	*arrest*	*Df(2L)escP3-0*	33A1-2	33E	
36AB	*blanks*	*Df(2L)H20*	36A8-9	36E3-4	
38E	*diaphanous*	*Df(2L)TW84*	37F5-38A1	39D3-E1	√
42A	*ms(2)42A*				√
42D	*ms(2)42D*	*Df(2R)pk78s*	42C1-7	43F5-8	
43C	*ms(2)43C*				
46AB	*ms(2)46AB*				
46C	*ms(2)46C*				
46C	*dispersed*				√
46F	*mulet*				
49C	*oxen*	*Df(2R)vg-C*	49B2-3	49E7-F1	
50D	*peanuts*				√
51D	*bocce*	*Df(2R)JP1* (1)	51C3	52F5-9	√
55A	*halley*	*Df(2R)Pc4*	55A	55F	
56E	*emmenthal*	*Df(2R)G100-L141* (2)	56D	56F	
57E	*trail mix*	*Df(2R)PuD17*	57B4	58B	
58F	*bellwether*				
60A	*ken and barbie*	*Df(2R)bwS46*	59D8-11	60A7	
61B	*pointless*				√
61CD	*ms(3)61CD*				
62A	*cueball*	*Df(3L)Ac14-8* (3)	61C3,4	62A8	√
63AB	*scratch*				√
65E	*ms(3)65E*				
66F	*boule*	*Df(3L)29A6*	66F3	67B1	√
68C	*pistachio*	*Df(3L)vin2*	67F2-3	68D6	
72D	*ms(3)72D*				
73D	*ms(3)73D*				
77B	*fumble*	*Df(3L)rdgC* (4)	77A1	77D1	
78D	*betel*	*Df(3L)Pc-MK*	78A3	79E1-2	
80	*ms(3)80*				
82C	*shank*	*Df(3R)Z-1* (5)	82A5-6	82E4	
82D	*bobble*	*Df(3R)6-7* (6)	82D3-8	82F3-6	
83B	*gorp*				√
84F	*seedless*				
85A	*capon*	*Df(3R)p13* (7)	84F2	85B	
85D	*ms(3)ry85D*				
86E	*thousand points of light*	*Df(3R)TE32* (8)	86E2-4	87C6-7	√
88B	*cashews*	*Df(3R)red1*	88B1	88D3-4	
88D	*effete*				√
89B	*ms(3)89B*				
89E	*twig*	*Df(3R)bxd100*	89B5-6	89E2-3	
90E	*ms(3)90E*				
91B	*fruitless*	*Df(3R)ChaM5*	91B3	91D1	√
95F	*jaguar*	*Df(3R)crbS87-5*	95F7	96A17-18	√
97D	*Rb97D*	*Df(3R)Tl-I*	97B	97E	
97EF	*goulash*	*Df(3R)Tl-P*	97A	98A1,2	
98B	*ms(3)98B*				
98D	*gruyère*				√
100E	*hephaestus*				
100EF	*ms(3)100EF*				

All deficiencies are referenced in LINDSLEY and ZIMM (1992) except as noted: (1) SAXTON *et al.* (1991); (2) LINDSLEY *et al.* (1972); (3) J. POSAKONY, personal communication; (4) STEELE and O'TOUSA (1990); (5) LETSOU *et al* (1991); (6) S. ALEXANDER and S. WASSERMAN, unpublished results; (7) K. KEMPHUES, personal communication; (8) GAUSZ *et al.* (1991).

[a] A checkmark under R (revertability) indicates a locus for which transposase-induced excision of the *P* element was carried out and male-fertile revertants were obtained; absence of a checkmark merely indicates that reversion was not attempted.

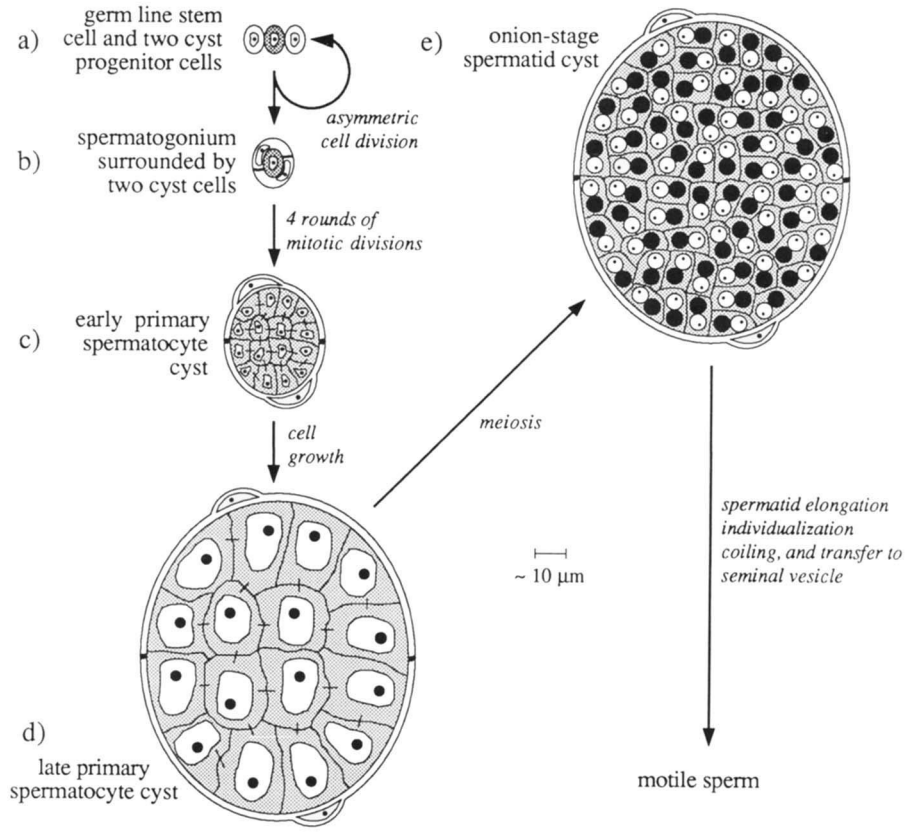

FIGURE 1.—Overview and schematic of key stages of spermatogenesis in *Drosophila melanogaster*. Structures in this figure are portrayed similarly but not exactly as they appear in phase contrast microscopy of unfixed testis contents. The two cyst cells surrounding each group of germ cells are shown for the sake of clarity but are not easily visualized by phase contrast. Nuclei are shown as open circles against a background of gray (germline cells) or white (somatic cyst cells) cytoplasm. Black spots in nuclei in a–d are nucleoli; black dots in nuclei in e are protein bodies. Structures are drawn approximately to scale. Spermatogenesis entails the differentiation of cells of both germinal and somatic origin. The precursors for these cells, the germline stem cells and the somatic cyst-progenitor cells, are attached to a specialized somatically-derived structure, the hub, at the tip of the testis (HARDY *et al.* 1979). At the hub, each stem cell is closely associated with two cyst progenitor cells (a). These three stem cells undergo unequal cell divisions: the parental stem cells remain attached to the hub, while the three daughter cells (one spermatogonium and two cyst cells) detach and initiate a program of differentiation (b). The transformation of a single spermatogonium to 64 sperm occurs within a thin envelope formed by these two cyst cells; this group of germline and somatically derived cells is defined as a cyst. Four rounds of mitotic division result in a cyst of 16 early primary spermatocytes interconnected by cytoplasmic bridges or ring canals (c). These spermatocytes enter a growth phase in which they undergo a 25-fold volume increase (d). During this growth phase a great deal of transcription takes place (OLIVIERI and OLIVIERI 1965); the bulk of these mRNAs are stored and not translated until after meiosis (BRINK 1968). The late primary spermatocytes enter meiosis, giving rise to 64 haploid spermatids. The nucleus in each spermatid reforms (open circle) and the mitochondria fuse to form the mitochondrial derivative or nebenkern (solid circle). At the onion stage, so-called because of the multilamellate appearance of the nebenkern in electron micrographs, both of these structures are closely associated and highly uniform in size and shape (e). Each young spermatid will be transformed into a mature sperm through a complex process of cytodifferentiation (TOKAYASU *et al.* 1972a,b). This process involves nuclear condensation as well as the dramatic elongation of the axoneme and the mitochondrial derivative to form the sperm tail. The final steps of spermatogenesis are individualization, the process by which each elongated spermatid becomes tightly invested in its own membrane, and coiling, which results in the cyst of 64 sperm being drawn to the base of the testis. During this coiling process, grossly abnormal sperm are segregated away and are subsequently degraded. The remaining sperm are transferred to the seminal vesicle for storage.

The *bocce* mutation is pleiotropic. Females homozygous for *boc* are semisterile. Furthermore, in *trans* to a deficiency, the *boc* mutation becomes semilethal and the defect in spermatogenesis becomes more severe. Defective spermatocyte cysts are similar to those seen in homozygotes; however, no postmeiotic cysts are found.

Mutations at the *cueball* (*cue*) locus in 62A, first identified by P. G. WILSON and M. T. FULLER (personal communication), cause an accumulation of spermatocytes with cytoplasmic abnormalities. Early spermatocytes are filled with small, refractile cytoplasmic inclusions (Figure 3d, arrows); late spermatocytes often contain up to three hook-shaped structures in the cytoplasm (Figure 3e, arrows).

The morphology of *cue* testes is also abnormal. Testes are short, with incomplete coiling. The sheath of pigment cells that covers the muscle layer surrounding the testis frequently peels away, particularly from the region near the tip. Fertility is also affected in females. The severity of phenotypes in both sexes is more extreme in *trans* to a deficiency; in females,

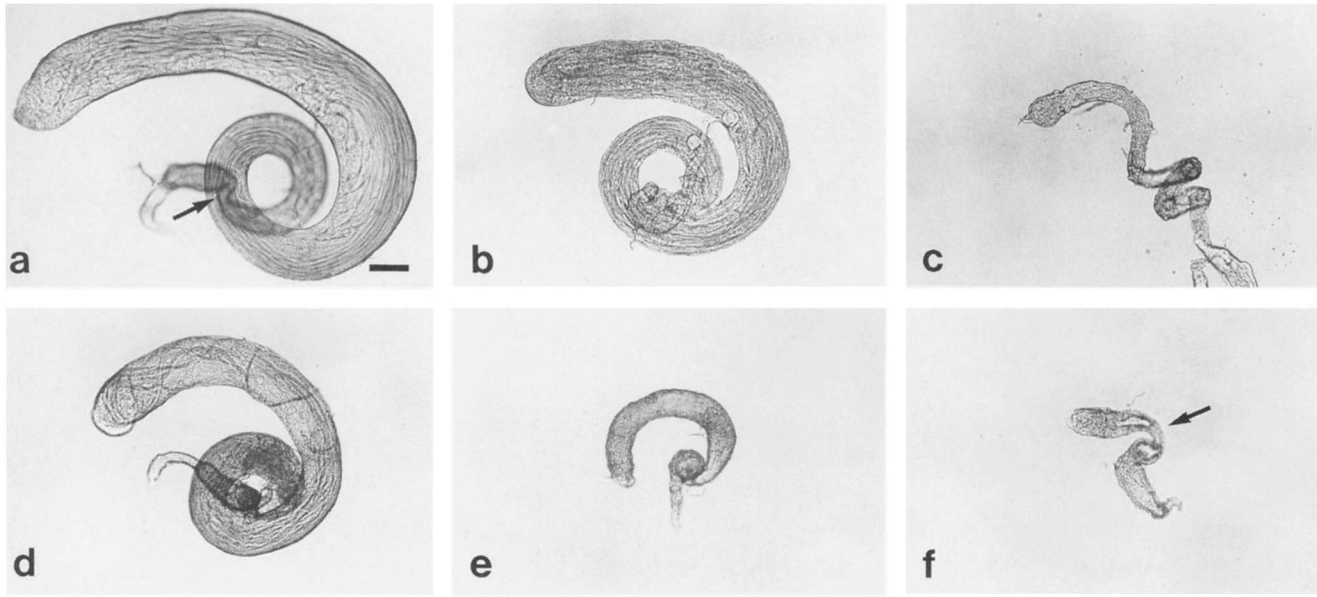

FIGURE 2.—Testis morphology of proliferation defect mutants. Testes are from newly eclosed males unless otherwise noted. Arrows at the base of the testis indicate junction between testis and seminal vesicle (shown only for a and f). Bar represents 100 μm. (a) *dia*/+ testis exhibiting wild type morphology. Proliferating cysts are present at the testis tip, while growing spermatocytes are found along the inside wall of the testis through the first coil. Elongated sperm tails can be seen extending from the base almost to the tip of the testis. (b) *dia*/*dia* testis, somewhat smaller than wild type, yet with some elongated cysts, as evidenced by the presence of sperm tails. (c) *dia*/*dia* testis, 5-day old male. Testis is very thin and appears devoid of germinal contents. (d) $chic^{11}/chic^{11}$ testis, slightly smaller than wild type, exhibiting some elongated sperm tails (e) $chic^{11}/Df(2L)GpdhA$ testis, much shorter than wild type, with severely reduced germinal contents. (f) testis from progeny of osk^{301}/osk^{301} mother. When raised at 18° such mothers give rise to viable progeny lacking pole cells (LEHMANN and NÜSSLEIN-VOLHARD 1986). The resulting germlineless adult testis is stunted.

gonads that appear to lack a germline are occasionally found.

Testes of males mutant for *scratch* (*stc*) at 63AB contain needle-shaped crystals in spermatocytes, as well as spermatids (Figure 3f). The crystals are similar in size, shape, and distribution to the proteinaceous crystals found in the testes of *XO* males (MEYER 1968; COX, WHITE and KIEFER 1976). In addition, meiosis in *scratch* males is defective. Nuclear size is variable (Figure 3f, arrows), as is the size of the spherical mitochondrial mass called the nebenkern (Figure 3f, arrowheads). These defects are also observed in *XO* males (LIFSCHYTZ 1987). Thus, the *scratch* insertion is a recessive-third chromosome mutation which results in a male-sterile phenotype similar to that seen in males lacking a *Y* chromosome.

The two alleles of *l(2)26Ab* cause degeneration of spermatocytes prior to completion of the growth phase (Figure 3g, arrowheads) and no meiotic products are observed. In addition, *l(2)26Ab* testes are shorter than wild type. The *l(2)26Ab* insertions, which map to 26A, are allelic to the lethal mutation *l(2)gdh-2* (KOTARSKI, PICKERT and MACINTYRE 1983). In *trans* to *l(2)gdh-2*, the *l(2)26Ab* insertions are male-sterile but viable, indicating that these male-sterile *l(2)26Ab* alleles do not represent a complete loss of function.

Whereas males homozygous for *fumble* (*fbl*) at 77B show no defects prior to meiosis, the *fbl* insertion in *trans* to a deficiency causes defects at earlier stages of spermatogenesis. As for *l(2)26Ab* homozygotes, *fbl*/*Df* males have short testes containing degenerating spermatocytes. In addition, with low penetrance, cysts with more than 16 mitotically dividing cells have been observed in 5-bromodeoxyuridine labelling experiments (P. GÖNCZY, unpublished results). When homozygous, *fumble* insertions result in meiotic division defects (see below). Given this range of phenotypes, classification of this mutant is conditional and awaits the generation of a null allele.

Meiotic entry defects: Mutations in two loci prevent entry into meiosis. In wild-type testes, the two meiotic divisions result in cysts of 64 round spermatids, which are found approximately one-third of the distance along the length of the testis (Figure 4, a and c). These spermatids then move toward the base of the testis, while sperm tails form and elongate until they span almost the entire testis length (Figure 4a). In mutants defective for meiotic entry, haploid cells are not formed and the absence of both unelongated and elongated spermatids is readily apparent through the testis sheath. The entire length of testes from these mutants is filled with spermatocyte cysts (Figure 4, b and d).

Spermatogenesis in males mutant for *pelota* (*pelo*), which maps to 30C, appears wild-type up to the 16-cell spermatocyte stage. Thereafter, *pelota* spermatocytes fail to undergo meiotic nuclear reduction, but nonetheless differentiate further. In mutant sperma-

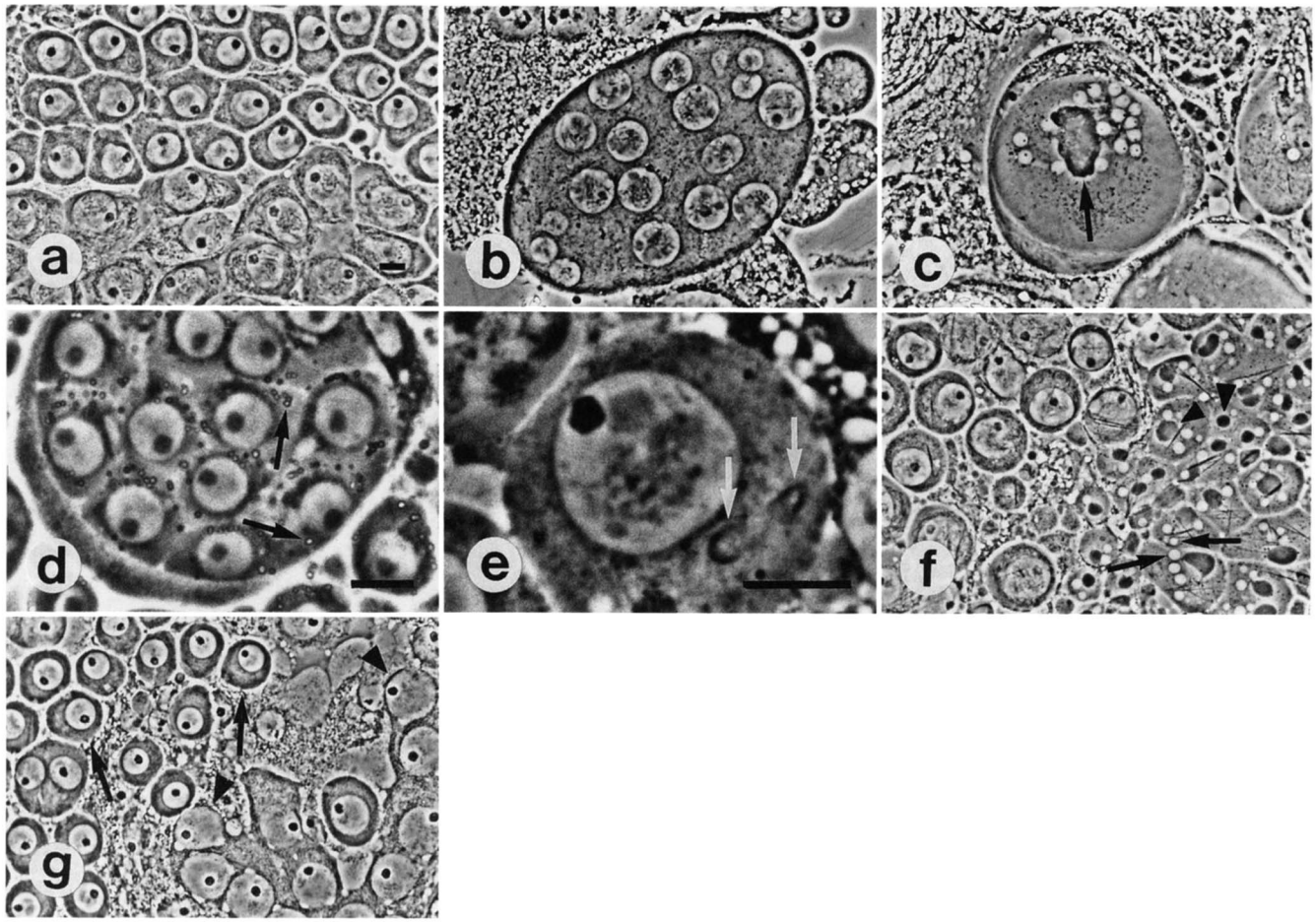

FIGURE 3.—Phenotypes of growth phase defect mutants, visualized by phase contrast microscopy of unfixed testis contents. Bars represent 10 μm; panels a, b, c, f and g are at the same magnification. (a) Wild-type spermatocytes have prominent nuclei, whose diameter increases during the growth phase (compare younger spermatocytes in top half of panel with more mature spermatocytes at bottom). (b) *boc/boc* spermatocyte cyst. Nuclei are of variable size, and the cytoplasmic organization apparent in the wild-type is lost, giving the appearance that cell boundaries are absent. The number of nuclei is somewhat variable, but is on average 16; at least 17 nuclei are visible in this cyst. (c) *boc/boc* spermatid cyst. Existence of a single giant mitochondrial mass (arrow) is indicative of a lack of cell boundaries. (d) cue^2/cue^2 early spermatocytes. Cytoplasm of most cells contains several refractile inclusion bodies (arrows). (e) cue^2/cue^2 late spermatocyte. Cytoplasm typically contains 1–3 hook-shaped structures (arrows). (f) *stc/stc* late spermatocytes and early spermatids. Needle-shaped crystals are prominent in late-stage spermatocytes as well as in spermatids. Meiosis is severely defective, resulting in nuclei (arrows) and nebenkern (arrowheads) of variable size. (g) $l(2)26Ab^5/l(2)26Ab^5$ spermatocytes. Young spermatocytes (arrows) appear normal, but degenerate prior to completion of the growth phase. Degenerating spermatocytes lyse, as evidenced by the loss of cytoplasm surrounding the nuclei (arrowheads).

tid cysts, the nuclei become pale and homogeneous, but remain as large as spermatocyte nuclei. The mitochondria fuse to form an abnormal, large nebenkern (Figure 4e). Little differentiation takes place beyond this stage, as very few elongated cysts are seen.

The male-sterile *pelota* phenotype is reminiscent of that of a mutation in *twine*, a Drosophila *cdc25* homolog (COURTOT *et al.* 1992; ALPHEY *et al.* 1992), certain heteroallelic combinations of *haywire* mutations (REGAN and FULLER 1990) and three *X*-linked male-sterile mutations (LIFSCHYTZ and HAREVEN 1977), in that aspects of postmeiotic differentiation occur in the absence of nuclear reduction. The *pelota* mutation also results in female sterility; *pelota* females have small ovaries and lay very few eggs. At 18°, *pelota* becomes semilethal and both the spermatogenesis and oogenesis phenotypes are more severe.

The phenotype of some cysts from males mutant for *boule* (*bol*) at 66F is very similar to that seen for *pelota*. Such cysts are comprised of 16 cells, each containing a large nucleus and nebenkern resulting from postmeiotic differentiation in the absence of meiosis. In other cysts, 16 large nebenkerne are associated with a greater number of variably sized nuclei. Thus, in this subset of cysts, aberrant nuclear division appears to take place in the absence of cytokinesis.

Meiotic division defects: Mutations in three loci result in defects first detectable during the meiotic divisions. Following wild-type meiosis, each spermatid nucleus appears as a pale sphere (Figures 1e and 5a). Next to each nucleus, and equal in size, lies a dark, spherical nebenkern. Since nuclear diameter in early spermatids correlates with chromosome content (GONZALEZ, CASAL and RIPOLL 1989), a defect in chromo-

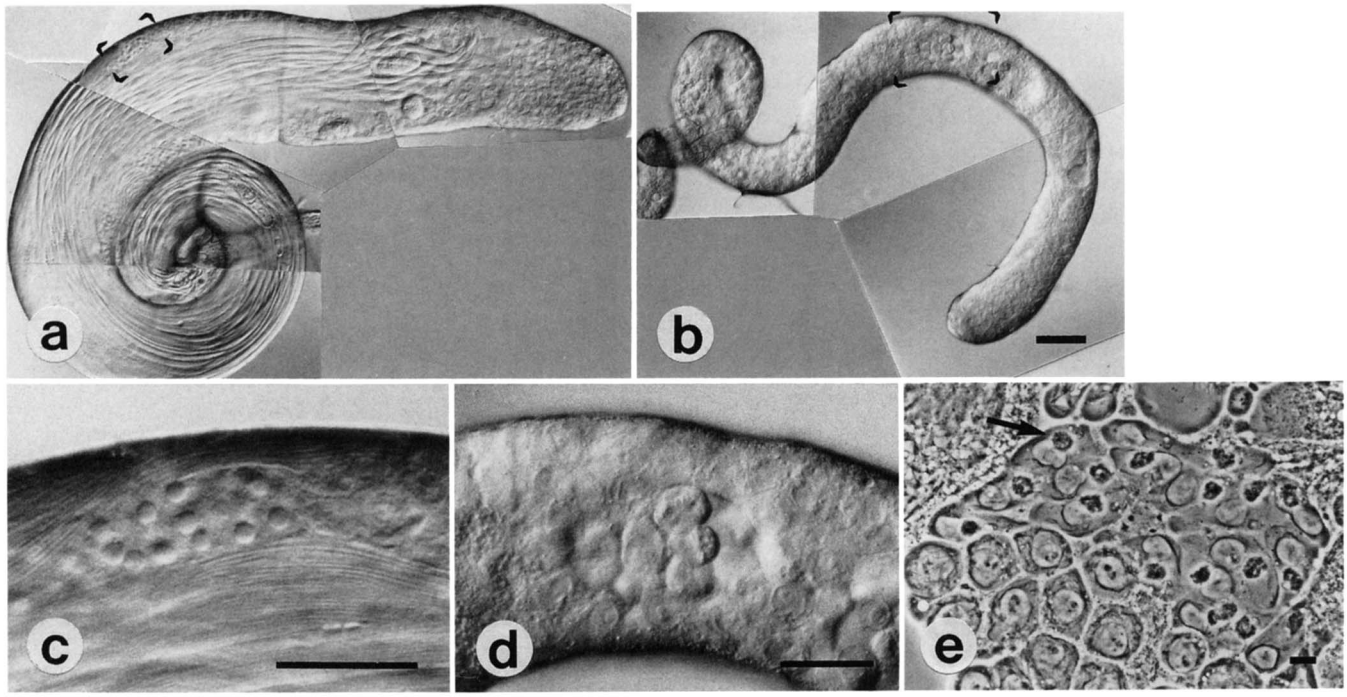

FIGURE 4.—Phenotypes of a meiotic entry defect mutant. Panels a-d viewed with Nomarski optics. Bar in b represents 100 μm (panel a is at same magnification); bars in c and d represent 50 μm; bar in e represents 10 μm. (a) *pelo*/+ testis with elongating spermatids; sperm tails can be seen stretching throughout the entire testis. (b) *pelo*/*pelo* testis filled with premeiotic cysts. No elongated cysts are present. (c) Higher magnification of framed region in panel a. Nuclei of wild-type unelongated spermatids, the immediate products of meiosis, appear as small spheres and are found approximately one-third of the distance along the length of the testis. Note the neighboring sperm tails, which belong to older cysts. (d) Higher magnification of framed region in panel b. Meiosis does not occur in the *pelo* testis and the lumen of the testis fills with large, spherical premeiotic germ cells. (e) contents of *pelo*/*pelo* testis, viewed in phase contrast. *pelo* spermatocytes mature fully, then form a nebenkern (arrow) adjacent to the nucleus.

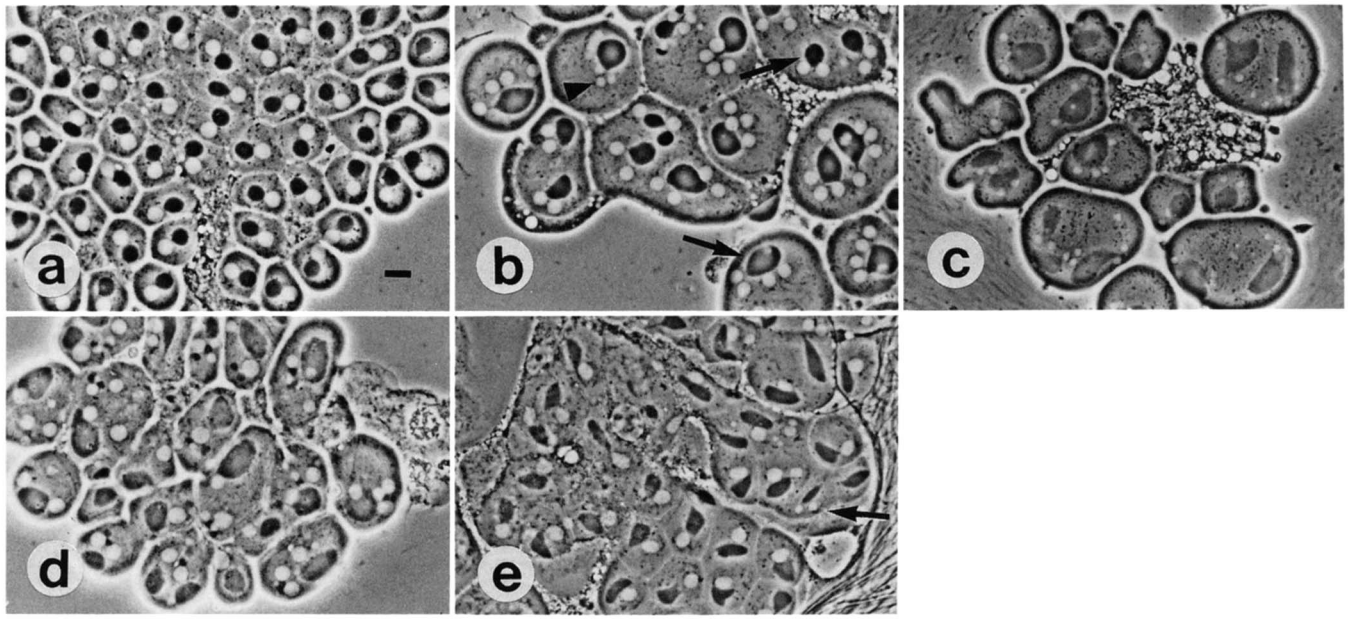

FIGURE 5.—Phenotypes of meiotic division defect mutants visualized by phase contrast microscopy of unfixed testis contents. Bar represents 10 μm. (a) wild-type spermatids. Each spermatid contains a single nucleus (appear as clear circles) and a single nebenkern (appear as dark circles) of uniform size. (b) *shk*/*shk* spermatids. Typically two or four nuclei of uniform size are associated with abnormally large nebenkern (arrows); some abnormally small nuclei are also present (arrowhead). (c) fbl^1/fbl^1 spermatids. Large, pale nebenkerne are associated with two to four very small nuclei of uniform size. (d) *dbf*/*dbf* spermatids. Both nuclei and nebenkern are variable in size. (e) *bob*/*bob* spermatids. Meiosis is mildly defective. Most spermatids appear normal but some contain abnormally small nuclei (arrow). The nebenkerne in this cyst appear variable in shape because the cyst as slightly older and the spermatids have begun to elongate.

some segregation manifests itself by variable nuclear size. Similarly, since inhibition of cytokinesis leads to the aggregation of the four nebenkerne into a single mass (LIEBRICH 1982), a defect in this process is apparent from a disruption in the normal one-to-one ratio between nebenkerne and nuclei. For mutations with defects in either chromosome segregation or cytokinesis, spermatid differentiation proceeds nonetheless and elongated cysts are produced.

The *shank* (*shk*) mutation at 82C results in abnormal spermatids. A single large nebenkern (Figure 5b, arrows) associated with two or four nuclei is indicative of a defect in cytokinesis. The nuclei in most spermatids have a size and shape similar to wild-type; other spermatids contain a large number of abnormally small nuclei (Figure 5b, arrowhead). A phenotype resembling that of *shank* has been observed for the male-sterile *four-wheel drive* locus (FULLER 1993). The *shank* mutation has less severe phenotypic consequences in *trans* to a deficiency.

Whereas *fbl/Df* males exhibit defects prior to meiosis (see above), testes from *fbl/fbl* males contain early spermatids in which groups of two or four nuclei are associated with a single, abnormally large nebenkern (Figure 5c). The *fbl* locus hence appears to be required for cytokinesis. The nuclei in many of these *fumble* spermatids are smaller than wild type, but are uniform in size. The uniform size of these micronuclei may indicate that they result from abnormal condensation rather than defective chromosome segregation. Nuclear defects persist during *fbl* spermatid differentiation; nuclei in later spermatids fail to change shape but appear instead as clear circular bodies throughout the length of elongating cysts. Such spherical nuclei are also seen among late stage spermatids in several other mutants from our collection (see Table 2 and Figure 6), as well as sterile males with *X*:autosome translocations (SHOUP 1967) and *tra2* males (BELOTE and BAKER 1983).

An insertion in *doublefault* (*dbf*) at 32A results in abnormally sized nebenkerne and nuclei in a high proportion of cysts (Figure 5d). Both cytokinesis and chromosome segregation may therefore be defective. Abnormal morphology is also apparent in elongating spermatids: the nuclei fail to change shape and are found scattered throughout the cysts.

The *bobble* (*bob*) mutation at 82D exhibits mildly defective spermatid cysts. Most spermatids are normal, but a few are aberrant, containing nuclei of variable size (Figure 5e, arrow). The lack of uniform nuclear size may reflect abnormal chromosome segregation. If so, however, a variable nuclear DNA content cannot of itself be responsible for the lack of motile sperm in *bob* males, since even complete absence of chromosomal content does not block production of functional sperm (LINDSLEY and GRELL 1969).

Postmeiotic differentiation defects: The majority of the male-sterile mutations result in defects in postmeiotic differentiation. Following completion of meiosis, wild-type haploid germ cells differentiate and elongate. The clustered spermatid nuclei are reduced in volume and transformed in shape from spheres to long, thin cylinders as they reach the base of the testis (Figure 6a). At the same time, an axoneme develops, along with the associated mitochondrial derivative. At the end of the differentiation program, the mature spermatozoa exit the testis and enter the seminal vesicle for storage. Mutants in this class successfully carry out meiosis, but produce no motile sperm for passage from the testis into the seminal vesicle.

For several mutants we have been able to detect defects in nuclear shaping or clustering by staining fixed testes with the DNA-binding dye Hoechst 33258. The *halley* (*hal*) mutation at 55A results in a failure of most spermatid nuclei to change shape; the clustered nuclei remain round even as they reach the base of the testis (Figure 6b, arrow). In addition, some of the nuclei are scattered (arrowhead). These defects are also detectable in testis squashes. Round nuclei are found in *halley* spermatid cysts (Figure 6e) at a stage when wild-type nuclei are no longer visible, and some nuclei are scattered throughout the length of the cyst (Figure 6e, arrows).

The *ms(2)46C* mutation results in nuclei that become cylindrical, as in wild-type, but are not fully condensed and fail to remain clustered (Figure 6c). In *ms(3)72D* homozygotes, the nuclei appear contorted into various shapes rather than extended in the normal configuration (Figure 6d). Other mutations with nuclear defects apparent in Hoechst staining are listed in Table 2.

Mutations in *emmenthal* (*emm*) at 56E and *gruyère* (*gru*) at 98D affect nebenkern morphogenesis, as visualized by phase contrast microscopy. Nebenkerne from *emm* males are highly vacuolated, but are otherwise normal in size and shape, as well as in their association with nuclei (Figure 6f, arrows). *gru* gives rise to a similar phenotype, but fewer nebenkerne are vacuolated. A number of *D. hydei* mutants with similar phenotypes have been described (HACKSTEIN *et al.* 1990).

Behavioral defects: Mutations in two loci result in behavioral sterility. Males mutant for the previously described *fruitless* (*fru*) locus at 91B exhibit aberrant courtship behavior, courting females and males indiscriminately (GILL 1963; GAILEY and HALL 1989). The *P[Z]* alleles are sterile as homozygotes or in *trans* to a deficiency for the locus, but do not appear to be null: unlike such a deficiency, they show some fertility in *trans* to an inversion in *fru*. In addition, homozygotes for both alleles lack most of the male-specific abdominal muscle, whereas males carrying some other allelic

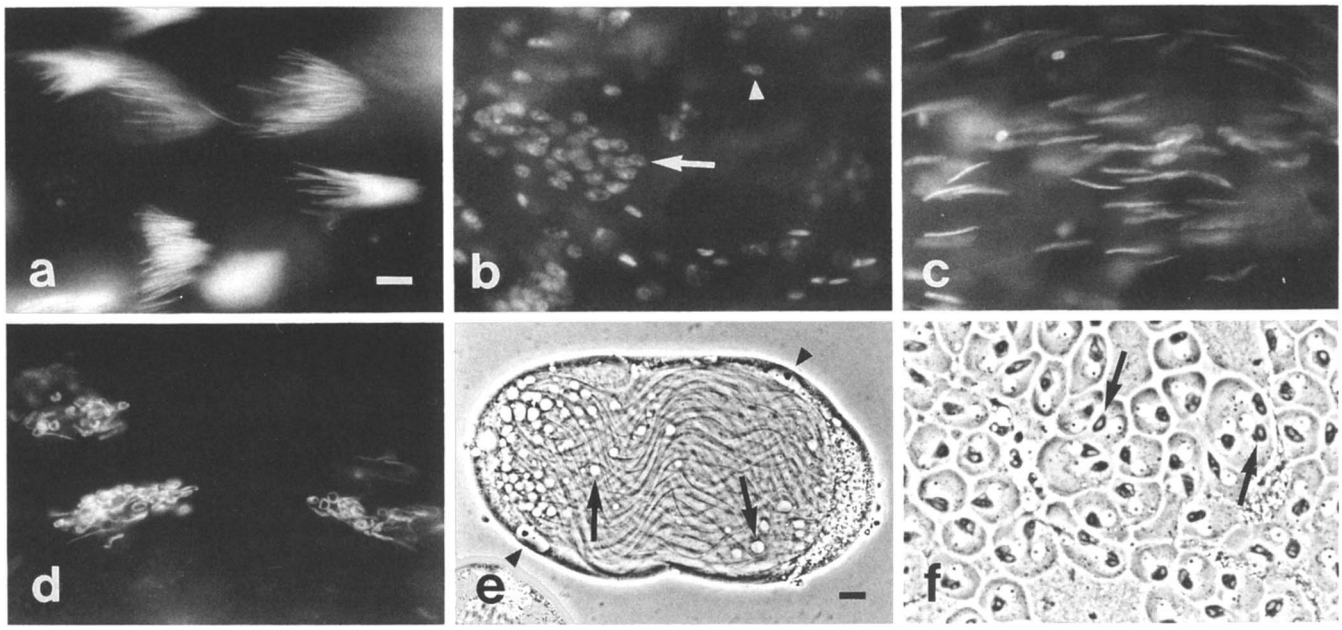

FIGURE 6.—Phenotypes of post-meiotic differentiation defect mutants. (a–d) Testes stained with Hoechst 33258. Photographs are of the base of the testis with the base toward the right. Phase contrast views of unfixed testis contents (e and f). For both sets of panels, bar represent 10 μm. (a) Elongated spermatid nuclei from *hal/+* testis exhibiting wild-type morphology. Several clusters of threadlike spermatid nuclei are visible (some clusters are out of focus). (b) Nuclei from *hal/hal* testis fail to elongate. Although most nuclei remain clustered (arrow), others are scattered (arrowhead). (c) Nuclei from *ms(2)46C/ms(2)46C* testis are completely scattered; these nuclei have elongated but are not fully condensed. (d) Nuclei from *ms(3)72D/ms(3)72D* testis adopt defective shapes but remain clustered. (e) *hal/hal* elongating spermatid cyst with round nuclei (appearing as clear spheres). Most nuclei are located at the head end of the cyst (left), but some are scattered throughout the cyst (arrows). [Note that cyst cell nuclei are easily seen in this intact cyst (arrowheads).] (f) Spermatids from *emm/emm* testis. Nebenkerne contain one or more vacuoles (arrows).

combinations lack the muscle entirely (GAILEY, TAYLOR and HALL 1991).

Males homozygous for either of the *cuckold* (*cuc*) alleles at 28A are semisterile. When individual pairs were observed for 30 min, *cuc* males failed to court or mate with virgin females, whereas heterozygous control males courted, and in most cases, mated during the observation period. Males and females homozygous for either allele have decreased longevity; almost all flies die within 8 days after eclosion. These results might suggest that mutations in any gene resulting in general metabolic debilitation would be male-sterile due to a failure to carry out courtship (see also CARTHEW and RUBIN 1990). We note, however, that the only two male-sterile mutants in our collection which do not court are allelic to one another.

Sperm transfer defects: Mutations at two loci result in structural barriers to copulation. These mutations cause partial or complete sterility, despite the presence of mature gametes. The *ken and barbie* (*ken*) mutation at 60A causes semilethality as well as an incompletely penetrant sterile phenotype. Sterile males and females lack external genitalia, but dissection frequently reveals the presence of internalized genitalia. In addition, aristae are sparse and unpigmented, giving them a blond appearance. The *twig* mutation at 89E is also pleiotropic, leading to unpigmented bristles, as well as a rotated anal-genital plate, in both males and females.

The seminal vesicles of males homozygous for the *pointless* (*ptl*) mutation at 61B contain wild-type levels of motile sperm. However, dissection of virgin females immediately after mating with *ptl* males reveals that few or no sperm are transferred to the female sperm storage organs.

Expression of enhancer trap reporter genes: Each of the *P* elements used to generate our male-sterile collection contains a *lacZ* enhancer trap, a bacterial reporter gene that can be brought under the control of regulatory elements near the site of insertion in the genome (O'KANE and GEHRING 1987). The pattern of expression of the *lacZ* reporter gene often reflects that of the disrupted locus and may therefore provide information about the domain of expression of a gene of interest before it is cloned (FASANO, CORÉ and KERRIDGE 1988; BIER *et al.* 1989; WILSON *et al.* 1989).

The pattern of expression of the *lacZ* enhancer trap was examined for the 77 male-sterile lines in which spermatogenesis was defective. In 71 of these lines, β-galactosidase expression was observed in specific subsets of somatic and germline cells of the testis (data not shown). We considered the possibility that some mutations might alter cell fate and that such alterations in cell fate would be revealed by a change in the

expression of the *lacZ* reporter gene in the mutants. We therefore looked for differences in the X-gal staining pattern of heterozygotes and homozygotes, but did not find any. Nor did we find that the expression of the reporter gene was restricted to the stages of spermatogenesis affected by the insertion.

We did find a striking enrichment for one expression pattern among the lines defective for spermatogenesis: staining in all germline cells of the testis. Among the 77 lines, 21 expressed β-galactosidase in the germline beginning in stem cells and persisting through the spermatid stage. By comparison, in an examination of 700 viable fertile inserts of the *P[lacW]* element, only two lines had expression throughout the adult male germline (GÖNCZY, VISWANATHAN and DINARDO 1992; P. GÖNCZY and S. DINARDO, unpublished results).

DISCUSSION

Spermatogenesis in Drosophila offers a variety of challenges and opportunities to developmental biologists. A range of fundamental processes, including cell-cell interaction, mitosis, meiosis, and morphogenesis, all occur in a stereotyped spatial and temporal pattern. Moreover, cells from all stages of spermatogenesis can be simultaneously observed in a single testis.

From a genetic standpoint, spermatogenesis is readily tractable, since sterile mutations can be easily generated, identified, and investigated. The presence or absence of a germline, the number and quality of mitotic and meiotic divisions, and the production of motile sperm can all be scored in the light microscope. Mutations provide the key to initiating molecular biological studies, a transition greatly facilitated by the use of mutations induced with single *P* elements.

Mutation frequencies: Male-sterile mutations were identified in our screen about one-tenth as frequently as lethal mutations. Given that the number of loci in *Drosophila melanogaster* that can mutate to lethality is about half the number of polytene bands (PERRIMON, ENGSTROM and MAHOWALD 1989), or approximately 2500, it can be calculated that at least 250 genes should mutate to male-sterility. In fact, as discussed by others (LINDSLEY and TOKAYASU 1980; PERRIMON *et al.* 1986; SCHÜPBACH and WIESCHAUS 1991), the number of genes required for fertility in either sex is likely to be much greater. Germline clones for a large fraction of zygotic lethal mutations on the *X* chromosome are sterile in females (PERRIMON, ENGSTROM and MAHOWALD 1989). Moreover, flies carrying temperature-sensitive lethal mutations often become sterile when shifted to non-permissive conditions as adults (SHELLENBARGER and CROSS 1979). Thus many loci required for fertility may mutate only rarely, or not at all, to give viable, sterile phenotypes.

Consideration of allele frequencies indicates that our collection does not represent the majority of loci mutating to a male-sterile phenotype. Only two loci, *chickadee* and *effete*, are represented by more than two alleles; both represent previously identified hotspots for *P* element insertion (BERG and SPRADLING 1991; COOLEY, VERHEYEN and AYERS 1992). Of the remaining loci, 53 out of 61 are represented by only a single allele. It is not surprising, therefore, that we did not obtain mutations in a number of autosomal loci known to mutate to male sterility, such as *bag-of-marbles*, *benign gonial cell neoplasm*, *ms(3)sa*, *twine* or β_2-*tubulin*, particularly since some loci may not be mutable with *P* elements. Nevertheless, we did isolate mutations in previously characterized male-sterile loci, including *arrest*, *chickadee* and *fruitless*. We also identified a mutation in a locus for an RNA binding protein (*Rb97D*), for which there were no known mutations (KARSCH-MIZRACHI and HAYNES 1993), as well as a male-sterile allele of a previously identified lethal complementation group (*l(2)gdh-2*).

Classification of male-sterile mutations: The range of male-sterile phenotypes we observed is similar to that seen in screens with chemical mutagens (LIFSCHYTZ 1987; HACKSTEIN *et al.* 1990; HACKSTEIN 1991). As in these previous screens, mutations affecting germline proliferation, spermatocyte growth and meiosis were relatively rare, while mutations affecting postmeiotic differentiation were the most common.

We classified male-sterile phenotypes with regard to the distinct stages that characterize spermatogenesis and grouped the mutations based on the earliest phenotypic deviation from wild-type differentiation observable in the light microscope. These classes are broad and surely encompass a number of cellular processes. Nevertheless, we believe that the current classification of mutants represents a good starting point. By focusing on loci in which mutations affect a given developmental stage, it should be feasible to dissect particular processes in spermatogenesis, despite the large number of genes required overall.

Two lines of evidence indicate that the earliest spermatogenesis phenotype detected in our mutants is a property of the locus rather than of particular mutant alleles. First, in the eight cases for which multiple alleles of a locus were isolated, all alleles belong to the same phenotypic category. Second, with one exception, when a mutation was placed in *trans* to a deficiency, the resulting defects in spermatogenesis fell into the same phenotypic class as observed for the homozygous *P* element. This was true even in those cases where the insertion was homozygous viable and female fertile, but became semilethal or female sterile in *trans* to the deficiency.

Some mutations block the transition from one stage to the next. In such cases, the mutation may define a

step in a dependent series along the spermatogenic pathway. For example, mutations in *l(2)26Ab* block development before the late spermatocyte stage. In the wild-type, therefore, subsequent processes depend on the execution of the step requiring *l(2)26Ab* function.

Spermatogenesis, is not, however, simply a linear series of dependent steps (LIFSCHYTZ 1987). For example, males lacking a *Y* chromosome exhibit defects in the spermatocyte growth phase, but still initiate meiosis and spermatid differentiation (MEYER 1968). A number of mutations in our collection also affect a given stage without blocking further development. For example, the *scratch* mutation results in the formation of crystals apparent at the growth phase as well as additional defects at meiosis. These phenotypes may reflect a complete loss of function in one of two or more parallel processes in the spermatogenic pathway. It is also possible, however, that the allele is hypomorphic and that a null mutation would result in a complete developmental arrest.

Sterile mutations in essential genes: A number of our male-sterile mutations affecting early stages of spermatogenesis disrupt gamete development in both sexes (Table 2). This is not unexpected, since there are many similarities in the early steps of male and female gametogenesis and thus many opportunities for shared gene function (SCHÜPBACH and WIESCHAUS 1991). Similarly, the fact that many of these mutations also affect viability presumably reflects the existence of functions common to cell growth or division in both the germline and the soma. Indeed, for females, at least, it appears that most genes which mutate to sterility are not exclusively required for gametogenesis (PERRIMON *et al.* 1986).

Male-sterile or female-sterile alleles of essential loci offer the opportunity to study phenotypes that are frequently more readily interpretable than the lethality that characterizes null mutations. For example, in the case of the Drosophila homolog of the epidermal growth factor (EGF) receptor, a requirement for the locus in dorsoventral pattern formation was apparent from characterization of female-sterile *torpedo* alleles (*Egfr*^{t}), but not zygotic lethal *faint little ball* mutations (*Egfr*^{f}) in the same locus (SCHÜPBACH 1987; PRICE, CLIFFORD and SCHÜPBACH 1989; SCHEJTER and SHILO 1989). Similarly, female-sterile alleles of *chickadee*, an essential locus (E. VERHEYEN and L. COOLEY, personal communication), revealed the participation of profilin, an actin-binding protein, in the anchoring of nurse cell nuclei within the developing egg chamber (COOLEY, VERHEYEN and AYERS 1992). Male-sterile *chickadee* alleles, which result in testes nearly devoid of germinal content, are likely to offer additional insight into profilin function.

Since *P* elements frequently insert upstream of coding sequences in the 5′ end of a gene, they may be particularly effective at inducing tissue or sex-specific alleles of essential loci. For example, many *P* element insertions in *chickadee* that are viable but sterile lie within an alternative 5′ exon (COOLEY, VERHEYEN and AYERS 1992).

Proliferation phase defects: Mutations that disrupt male germline proliferation provide an opportunity to begin to dissect processes of inherent interest in spermatogenesis: the control of stem cell identity, the synchronization of germline and somatic stem cell divisions, and the regulation of the number of mitoses.

Germline proliferation is affected by mutations in two of our male-sterile loci, *chickadee* and *diaphanous*. The phenotypes of these mutations suggest that these loci act prior to the growth phase. For *chic*, a proliferation defect has been demonstrated directly by an examination of 5-bromodeoxyuridine labeling in mutant and wild-type testes (P. GÖNCZY and S. DINARDO, unpublished results). For *dia*, null alleles are lethal at the pupal stage and produce aberrant mitotic figures in larval brains (D. CASTRILLON and S. WASSERMAN, unpublished results). Since *P* element mutations in either gene lead to a loss of germline cells in the testis, *chic* and *dia* are complementary in phenotype to *benign gonial cell neoplasm* and *bag-of-marbles*, mutations that cause an overproliferation of germline cells (GATEFF 1982; MCKEARIN and SPRADLING 1990). It remains to be proven whether mutations in these loci affect cell fate determination or the subsequent execution of the mitotic cell cycle.

Meiotic entry defects: Male-sterile mutations defective for entry into meiosis are also of experimental interest. Whereas the regulation of the meiotic cell cycle has been studied extensively in yeast (MALONE 1990), much less is known about this process in multicellular organisms. Although gametogenesis in flies has been studied more extensively in females than in males, spermatogenesis offers a significant advantage for the study of meiosis. The two meiotic divisions in spermatocytes occur in rapid succession in a discrete portion of the adult testis. In contrast, meiosis in females begins in the nuclei of pro-oocytes but is not completed until after fertilization, at least three days later.

The premeiotic cysts found in testes from *pelota* males undergo incomplete chromosome condensation and thus have arrested at the earliest steps of meiosis (C. EBERHART and S. WASSERMAN, unpublished results). Nevertheless, in *pelota* males, cells from these arrested cysts form a nebenkern, a mitochondrial form normally found only in postmeiotic spermatids. This uncoupling of meiosis and postmeiotic differentiation has also been observed for *twine*, as well as several other male-sterile mutations (ALPHEY *et al.*

1992; COURTOT *et al.* 1992; LIFSCHYTZ and HAREVEN 1977; REGAN and FULLER 1990).

Some mutations that we have placed into the growth phase class may also define genes required for entry into meiosis. For example, in *l(2)26Ab* mutant testes, maturing spermatocytes degenerate and no meiotic products are observed. The possibility exists that germ cell degeneration is a secondary consequence of an alteration in cell fate due to the absence of a signal required for initiation of meiosis.

Prospects for further genetic analysis of spermatogenesis: It is noteworthy that mutations in which there is an underproliferation of the germline, or a degeneration of the germline following developmental arrest, alter testis morphology. Testes from males homozygous for mutations in *diaphanous*, *chickadee*, *bocce*, *cueball*, *boule* and *l(2)26Ab* are substantially reduced in size. Mutations in *bag-of-marbles* and *benign gonial cell neoplasm* also result in small testes. That the size of the testis is highly correlated with the fate of germline cells is also evident in the tiny testes from the germlineless progeny of *oskar* females.

The short or shriveled testis phenotype could greatly facilitate the isolation of mutations in loci required for germline proliferation and growth. This phenotype is immediately obvious upon dissection of homozygous males, as is the absence of elongating cysts in the testis. Thus one could screen for mutations in genes required for early stages of spermatogenesis without the need for labor-intensive fertility tests.

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Note: A representative allele for each of the male-sterile loci described in this paper is available from the Bloomington *Drosophila* Stock Center.

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