Probing spermatogenesis in *Drosophila* with P-element enhancer detectors

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Summary

Formation of motile sperm in *Drosophila melanogaster* requires the coordination of processes such as stem cell division, mitotic and meiotic control and structural reorganization of a cell. Proper execution of spermatogenesis entails the differentiation of cells derived from two distinct embryonic lineages, the germ line and the somatic mesoderm. Through an analysis of homozygous viable and fertile enhancer detector lines, we have identified molecular markers for the different cell types present in testes. Some lines label germ cells or somatic cyst cells in a stage-specific manner during their differentiation program. These expression patterns reveal transient identities for the cyst cells that had not been previously recognized by morphological criteria. A marker line labels early stages of male but not female germ cell differentiation and proves useful in the analysis of germ line sex-determination. Other lines label the hub of somatic cells around which germ line stem cells are anchored. By analyzing the fate of the somatic hub in an agametic background, we show that the germ line plays some role in directing its size and its position in the testis. We also describe how marker lines enable us to identify presumptive cells in the embryonic gonadal mesoderm before they give rise to morphologically distinct cell types. Finally, this collection of marker lines will allow the characterization of genes expressed either in the germ line or in the soma during spermatogenesis.

Key words: spermatogenesis, *Drosophila melanogaster*, P-element enhancer detectors.

Introduction

Spermatogenesis in *Drosophila melanogaster* is an elaborate differentiation program amenable to analysis in the context of the whole organism. The sequence of cellular events occurring during male gametogenesis has been extensively described at the ultrastructural level by electron microscopy (reviewed by Lindsley and Tokuyasu, 1980). All morphologically distinct cell types and stages present in testes have been recognized. It has been shown that differentiation of the germ cells occurs within a space delimited by two somatic cells, the cyst cells. Gametogenesis hence entails close contact and developmental coordination between cells derived from two distinct embryonic lineages, the germ line and the somatically derived gonadal mesoderm.

The genetic analysis of spermatogenesis began with the discovery that the Y chromosome, although dispensable for viability, is absolutely required for male fertility (Bridges, 1916). Subsequent studies uncovered loci on other chromosomes that are required for spermatogenesis (reviewed by Lifschytz, 1987). A few male-sterile mutants provide valuable information about particular aspects of the differentiation program. For instance, mutations in either *benign-gonial-cell-neoplasm* (*bgcn*) or *bag-of-marbles* (*bam*) result in deregulated mitotic proliferation of early germ cells (Gateff, 1982; McKearin and Spradling, 1990). These genes could therefore encode proteins that control the stem cell or mitotic division program. Other mutants are blocked before entry into meiosis and their analysis could provide clues about the commitment to this key step in gametogenesis (Lifschytz, 1978; N. Wolf, P. Wilson and M. Fuller, personal communication; D. Castrillon and S. Wasserman, personal communication; P.G., S.V. and S.D., unpublished data). Finally, mutations in the gene encoding β2-tubulin, a structural protein specific to spermatogenesis, alter microtubule-based processes during meiosis and in postmeiotic germ cells (Kemphues et al., 1982). Second site mutations that fail to complement certain β2-tubulin alleles may identify genes encoding other components of the complex assembly process occurring in haploid germ cells (Regan and Fuller, 1988; Green et al., 1990).

Notwithstanding the analysis of these loci, little is known about the genetic circuitry controlling the proper execution of spermatogenesis. This lack of knowledge stems from the very large number of loci that can mutate to cause male sterility, as well as from their pleiotropy (reviewed by Lifschytz, 1987). Thus,
the majority of a series of male-sterile mutations proved to be either weak alleles or spermatogenesis-specific alleles of vital genes (Lifschytz, 1978; Lifschytz and Yakobovitz, 1978; Geer et al., 1983; Dybas et al., 1983). This could be a reflection of the sensitivity of the developing germ cells to the alteration of basic cellular functions. It could also signify that many products required for spermatogenesis are also needed in other developmental processes. The large number of male-sterile mutants and their pleiotropy renders a thorough genetic analysis quite laborious. Ordering gene action along a developmental pathway can be difficult without knowing the null phenotype for the gene in question. In the case of male-sterile mutants, obtaining null alleles might often yield lethal mutations whose phenotype during spermatogenesis could be studied only in mosaic animals. In addition, choosing particular male-sterile mutants on which to focus has proved difficult because most of them do not display a tight arrest phenotype (see Lifschytz, 1987).

The limitations associated with a classical mutant analysis argue for a complementary approach to study this developmental pathway. We, therefore, sought to analyze spermatogenesis in *D. melanogaster* with P-element enhancer detectors (O’Kane and Gehring, 1987; Bellen et al., 1989; Bier et al., 1989). Enhancer detectors contain a weak promoter fused to the lacZ reporter gene. Upon insertion of the P-lacZ element in the genome, the weak promoter can be brought under the influence of a neighbouring enhancer, resulting in spatially and temporally restricted β-galactosidase activity. It has been shown that several of the enhancers detected in this manner control the transcription of a neighbouring gene in a similar pattern (Fasano and Kerridge, 1988; Bier et al., 1989; Wilson et al., 1989).

P-element enhancer detectors can be utilized in different ways to probe spermatogenesis. First, they can serve to generate male-sterile lines by insertional mutagenesis. Compared to a classical approach, this provides the advantage of indicating where the mutated gene might be normally expressed. This is especially useful since male-sterile mutants are not easily categorized based on their phenotype alone. Second, enhancer detection is the method of choice to identify genes that play a role in spermatogenesis but that are not easily recovered in male-sterile screens. Thus, lethal P-insertion lines can be examined as heterozygotes to determine whether the essential gene in question is expressed in male gonads. Viable and fertile lines expressing lacZ during spermatogenesis can serve to identify genes that are redundant in function or whose mutant phenotype is not male-sterility. Moreover, viable and fertile lines can also identify genes that are essential for male fertility if the location of the insertion has not led to disruption of gene function. Lastly, enhancer detector lines provide indispensable molecular markers for each of the labeled cell types and stages during spermatogenesis. Such marker lines are crucial in following the fate of specific cells in wild-type and in different mutant backgrounds.

We have begun to investigate spermatogenesis in *D. melanogaster* by looking at patterned lacZ expression in homozygous viable and fertile enhancer detector lines. We describe marker lines that label germ cells, somatic cyst cells and all other somatic cells present in testes. This series of lines provides an entry point for characterizing genes expressed either in the germline or in the soma during male gametogenesis. Particular lines label germ cells or cyst cells in a stage-specific manner during their differentiation program. These expression patterns reveal transient identities for the somatic cyst cells that had not been recognized by ultrastructural criteria. We also describe how the ability to follow the fate of specific cells is critical in investigating gonadogenesis, and in probing interactions between germ line and soma during spermatogenesis.

### Materials and methods

**Enhancer detector lines**

Over 700 viable and fertile autosomal P-element enhancer detector lines were generously provided for us by the laboratories of Yuh-Nung and Lily Jan (Bier et al., 1989; 500 lines, preselected against those with a staining pattern in the embryonic nervous system), Margaret Fuller and Matthew Scott (150 lines) and Judy Kassis (Kassis, 1990; 60 lines). One line described in this paper (line 254, Fig. 6A through 6G) is homozygous lethal. We also included in this report the staining pattern of one male-sterile line (line ms-985, Fig. 5C). This line has been identified in our laboratory from a collection of enhancer detector lines generously provided for us by John Merriam and the *Drosophila* laboratories at UCLA.

All lines in this report except line 254, Fig. 6A through 6G, carried the P-lacW construct described in Bier et al. (1989). Upon lacZ expression, these lines gave rise to nuclear X-gal stain in premeiotic germ cells (see for instance Fig. 2B) and in somatic cells (see for instance Fig. 4B), as expected from the presence of the nuclear targeting signal upstream of the lacZ reporter gene. β-galactosidase was present in both the nucleus and the cytoplasm in postmeiotic germ cells, thereby showing up in the elongating sperm tails (see Fig. 2A and 2B, arrowhead). This is probably due to the reshaping of the spermatid nucleus whose diminished volume might not be able to retain the nuclear targeted β-galactosidase efficiently.

A few lines carried constructs with sequences from the engrailed gene, including a fragment from the engrailed promoter, fused to the lacZ reporter gene with an AUG codon (Kassis, 1990). In addition to the engrailed-specific patterns discussed in Kassis (1990), many of these lines had additional expression patterns dependent on the insertion site in the genome, effectively behaving as enhancer detectors. Owing to the lack of a nuclear targeting signal, lines expressing lacZ gave rise in this case to cytoplasmic X-gal stain (line 254 in this report, Fig. 6A through 6G).

The majority of the enhancer detector lines also expressed lacZ in other cells in the adult fly. However, since this did not interfere with their use as marker lines to probe spermatogenesis, the characterization of expression patterns outside the gonads were not pursued further.

We learned how to recognize better the different cell types present in testes during the course of this analysis. Subtle expression patterns may have been disregarded in the initial phase of this study. In addition, the lines examined have been preselected as indicated above. Thus, the frequency of
occurrence for a particular staining pattern could be only approximated, and is indicated in the text when relevant.

**X-gal staining of male gonads**

Testes from 1-day-old adults were dissected in *Drosophila* Ringer's solution, transferred to microtiter plates and fixed for 15 minutes in 1% glutaraldehyde (Fluka); 50 mM sodium cacodylate. The tissues were rinsed three times with staining buffer (7.2 mM Na$_2$HPO$_4$, 2.8 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 0.15 M NaCl), left at room temperature for 30 minutes and then incubated at 37°C for 12 to 16 hours in staining buffer plus 5 mM each of potassium ferro- and ferri-cyanide and 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The tissues were washed three times in PBS plus 1 mM EDTA, dehydrated in ethanol solutions of increasing concentrations and mounted in 2:1 Canada Balsam:methyl salicylate.

**Reagents for indirect immunofluorescence**

The rabbit anti-β-galactosidase polyclonal antibody was purchased from Cappel. The rabbit anti-β-tubulin polyclonal antibody was a gift from Elizabeth Raff and is described in Kimble et al. (1989); the polyclonal antibody was preadsorbed at its final dilution against an equal volume of fixed 0-3 h embryos (Kimble et al., 1989). The fasciclin III monoclonal antibody was a gift from Danny Brower and is described as DA.166 in Brower et al. (1981). The β-galactosidase monoclonal antibody was a gift from Alfonso Martinez-Arias. All rhodamine- and fluorescein-conjugated secondary antibodies were purchased from Jackson Laboratories. They were preadsorbed at their final dilution for 2 hours against an equal volume of fixed embryos from an overnight collection.

**Indirect immunofluorescent anti-β-tubulin and anti-β-galactosidase staining of male gonads**

Male gonads from third instar larvae (line 600, see results) were dissected in PEM (0.1 M Pipes, 1 mM MgCl$_2$, 1 mM EGTA, pH 6.9) and fixed for 20 minutes in 4% formaldehyde (EM grade; Polysciences) in PEX (PEM plus 0.1% Triton X-100). The tissues were rinsed in PEX, washed twice for 10 minutes in PBX (PBS plus 0.1% Triton X-100), and blocked for 90 minutes in PBX-2 (PBX plus 2% normal goat serum; Vector). The gonads were then incubated overnight at 4°C with 1:100 rabbit anti-β-tubulin and 1:50 mouse anti-β-galactosidase primary antibodies in PBX-2, and washed three times for 30 minutes in PBX at room temperature. The tissues were then incubated for 60 minutes at room temperature with 1:300 rhodamine-conjugated donkey anti-rabbit and 1:300 fluorescein-conjugated goat anti-mouse secondary antibodies in PBX-2, and washed three times for 30 minutes in PBX and for 30 minutes in PBS. Stained larval gonads were mounted in a drop of Fluoromount-G (Fisher) under a bridged coverslip.

**Indirect immunofluorescent anti-fasciclin III and anti-β-galactosidase staining of male gonads**

Fasciclin III antigen is sensitive to fixation and best results are obtained when staining is performed on unfixed tissue (Brower et al., 1981). This double-labeling experiment therefore involved two successive reactions: firstly staining for fasciclin III, performed on live tissue, followed by fixation and a second staining for β-galactosidase. Testes from 1-day-old adults (line 254, see results) were dissected in *Drosophila* Ringer's solution, incubated for 45 minutes at 37°C with 1:500 mouse anti-fasciclin III antibody in PBT-2 (PBS plus 0.1% Tween-20 and 2% normal goat serum), and washed four times for 5 minutes at 4°C in PBT (PBS plus 0.1% Tween-20). The tissues were then incubated for 30 minutes at 37°C with 1:300 rhodamine-conjugated goat anti-mouse antibody in PBT-2, washed four times for 5 minutes at 4°C in PBT, and fixed in 4% formaldehyde in PBT for 30 minutes at 4°C. The remainder of the experiment was carried out at room temperature. The tissues were washed three times for 5 minutes in PBT, incubated for 45 minutes in 1:5000 rabbit anti-β-galactosidase antibody in PBT, and washed three times for 20 minutes in PBT. The tissues were then incubated for 45 minutes with 1:300 fluorescein-conjugated donkey anti-rabbit antibody in PBT-2, washed three times for 20 minutes in PBT, counterstained for 2 minutes in 1 μg/ml Hoechst 33258 (Sigma) in PBT, rinsed with PBT and washed for 20 minutes in PBS. The apical tip of the testis was dissected and placed in a drop of Fluoromount-G under a coverslip.

**Immunocytochemistry**

Embryos (line 254, see results) were collected for 2 hours and aged for 13 hours at 25°C, then permeabilized, fixed and devitellinized as described (Mitchison and Sedat, 1983). The embryos were stored in 100% methanol at −20°C for several days. Prior to rehydration, they were treated for 15 minutes with 3% H$_2$O$_2$ in methanol to block endogenous peroxidase activity. Testes from 1-day-old adults (line 57, see Results) and agametic gonads (line 254, see results and below) were dissected in *Drosophila* Ringer's solution, fixed for 20 minutes in 4% formaldehyde in PBX and washed three times for 10 minutes in PBX. The immunocytochemistry procedure was a modified version of that described in Kellerman et al. (1990). The samples were blocked for 90 minutes in Blotto (5% powdered milk in PBX) and incubated overnight at 4°C with 1:1000 (embryos) or 1:5000 (testes and agametic gonads) rabbit anti-β-galactosidase antibody in Blotto. The rest of the experiment was carried out at room temperature. The samples were washed three times for 10 minutes in PBX, incubated for 60 minutes with 1:400 biotin-conjugated goat anti-rabbit antibody (Vector) in Blotto, and washed three times for 10 minutes in PBX. They were then incubated for 30 minutes with 1:500 horseradish peroxidase-conjugated streptavidin (Chemicon) in Blotto, washed three times for 10 minutes and once for 30 minutes in PBX. Diaminobenzidine (Polysciences) immunocytochemistry was carried out as described in Kellerman et al. (1990). Finally, the samples were counterstained for 2 minutes in 1 μg/ml Hoechst 33258 in PBX, rinsed with PBX, washed for 20 minutes in PBS and mounted in a drop of glycerol.

**Obtaining agametic males carrying P-lacZ**

Homozygous oskar$^{301}$ (Lehman and Nüsslein-Volhard, 1986) female virgins were collected, aged at 18°C for four days and crossed to males carrying P-lacZ (heterozygous males from line 254, see results). At 18°C, osk$^{301}$ mothers give rise to viable progeny that lack pole cells, resulting in flies with agametic gonads.

**Microscopy and photography**

The samples were examined with a Nikon Optiphot microscope equipped with Nomarski optics and epifluorescence. X-gal stains and immunocytochemistry were viewed with bright-field optics for low magnifications (see for instance Fig. 2A) and Nomarski optics for higher power views (see for instance Fig. 2B), and photographed with Kodak Ektar 25 film. Indirect immunofluorescence was photographed with Kodak Tech Pan film set at ASA 800 for rhodamine and ASA 125 for fluorescein and Hoechst. The phase-contrast view of a live adult testis (Fig. 1A) was photographed with Kodak Pan-X film.
Results

Spermatogenesis in Drosophila melanogaster

Each adult testis comprises all stages of spermatogenesis displayed on a developmental gradient, with the earliest cells located at the apical end (Fig. 1A, api) and the latest at the terminal end (Fig. 1A, ter) of the gonad. The essential stages of this 10 day differentiation program (reviewed by Lindsley and Tokuyasu, 1980) will be briefly reviewed below in order to place the lacZ expression patterns into a meaningful context.

Five to nine germ line stem cells and about twice as many somatic cyst progenitor cells are anchored around a hub of somatic cells (Fig. 1B, hub) at the apical tip of the adult testis. A germ line stem cell (Fig. 1B, ste) and two neighbouring somatic cyst progenitor cells (Fig. 1B, cyp), also acting as stem cells, divide asymmetrically (Fig. 1B, asy). This yields a primary gonial cell (Fig. 1B, spg) and two cyst cells (Fig. 1B, csc), respectively. The two cyst cells and the corresponding enclosed germ cell form a cyst, the fundamental unit of this differentiation program.

The primary gonial cell then undergoes four mitotic divisions (Fig.1B, mit), while the cyst cells no longer divide. This yields a cyst of 16 primary spermatocytes (Fig. 1B, spc), interconnected by ring canals as a result of incomplete cytokinesis. The spermatocytes then enter an extended growth phase (Fig. 1B, gro), after which they undergo the two meiotic divisions (Fig. 1B, mei). The 64 haploid spermatids (Fig. 1B, spt) then initiate a program of dramatic morphological changes (Fig. 1B, mor) in which virtually all cellular organelles are restructured and the flagellar tail is assembled. At the same time, the two cyst cells become structurally distinct. The head-cyst cell (Fig. 1B, cyh) surrounds and interdigitates with the sperm heads whereas the tail-cyst cell (Fig. 1B, cty) elongates along with the growing sperm tails. At the completion of spermatid differentiation, the head-cyst cell is entrapped by a specialized epithelial cell (Fig. 1B, tec) at the base of the testis. The process of individualization follows, during which single spermatozoa become invested in their own membrane. Coiling of the sperm bundle ensues, followed by release of motile spermatozoa (Fig. 1B, spz) in the seminal vesicle.

LacZ expression

We first examined testes of flies that do not contain an enhancer detector to assess levels of eucaryotic β-galactosidase. No X-gal staining was observed, except in occasional degenerating cysts and in waste bags, which contain superfluous cytoplasmic material released during the final stages of spermatid differentiation (see for instance Fig. 6A, solid and outlined arrowheads). The low level and sporadic nature of this endogeneous activity could not be confused with the reproducible expression patterns generated by individual enhancer detector lines. Moreover, eucaryotic β-galactosidase could easily be distinguished from lacZ expression by using an antibody directed specifically against the bacterial β-galactosidase (see for instance Fig. 5F, arrowheads).

We then examined male gonads in homozygous viable and fertile P-element enhancer detector lines (see Materials and methods). The frequencies of staining patterns in the germ line and the soma (excluding the epithelial cells from the sheath connecting the testis to the seminal vesicle) were determined by close examination of 120 lines. 55% expressed lacZ in the germ line only, 5% in the soma only, while 13% labeled both germ cells and somatic cells. 27% did not give rise to any staining pattern in male gonads.

From over 700 enhancer detector lines examined, we chose about fifty as molecular markers for the different cell types and stages of spermatogenesis. A description of selected lines follows.

Germ line

Most lines labeling the germline initiated lacZ expression during the spermatocyte growth phase and displayed β-galactosidase activity until after meiosis (Fig. 2A). Germ cells could be easily identified by their characteristic morphology and numbers. X-gal-positive cells in the growth phase region were typical of maturing spermatocytes, with a large nucleus and prominent nucleolus (Fig. 2B, arrow). X-gal positive cells were organized in groups of 16 large cells up to meiosis (Fig. 2C, arrow) and 64 smaller cells after that (Fig. 2C, arrowhead). Postmeiotically, X-gal positive nuclei became elongated and eventually rod-shaped (Fig. 2D, arrow), as expected from maturing spermatids undergoing nuclear reshaping. The germline nature of the labeled cells was further confirmed by crossing two such marker lines to oskar301 (osk301) females (Lehman and Nüsslein-Volhard, 1986). Progeny resulting from these crosses were agamic and carried a copy of the enhancer detector. As expected, lacZ expression was absent from these agamic testes (data not shown).

Other lines exhibited a staining pattern in germ cells that was restricted to particular stages of differentiation. Three expressed lacZ in stem cells and the mitotic phases of germ cell differentiation, but not in subsequent stages (Fig. 3A and data not shown). One of these labeled germ cells in the early stages of spermatogenesis (Fig. 3A), but not in the corresponding stages of oogenesis (Fig. 3B, arrow), characterizing it as an early marker of male germ cell identity. Another line that expressed lacZ in both early and later stages of male gametogenesis also failed to stain early female germ cells and was found to map to the same cytological position (data not shown).

Cyst cells

We identified marker lines that stain the somatic cyst cells (Fig. 1B, cye). Several expressed lacZ starting in the growth phase region and in all cyst cells past that stage (Fig. 4A). The lacZ-expressing cells were identified as cyst cells because each cyst of developing germ cells was associated with two X-gal-positive nuclei at its periphery (Fig. 4B, arrows), the number and location...
Fig. 1. (A) Phase contrast view of an adult testis. The apical end (api) is left in all figures unless otherwise noted; ter: terminal end. Bar=50 μm. (B) Schematic representation of 5 stages of spermatogenesis. Arrows pointing to part (A) indicate where approximately in the testis each stage begins; cells are displaced in an apical-to-terminal direction as they mature within each stage. Germ line stem cells and somatic cyst progenitor cells are anchored around a hub of somatic cells (hub) at the apical tip of the testis. Only one germ line stem cell (ste) and two cyst progenitor cells (cyp) are represented for clarity. asym: asymmetric divisions of a germ line stem cell and two neighbouring cyst progenitor cells give rise to one primary gonial cell (spg) and two cyst cells (cyc), respectively. mit: the spermatogonial cell undergoes four mitotic divisions, while the cyst cells no longer divide. gro: the resulting 16 spermatocytes (spc) grow dramatically. mei: the two meiotic divisions occur. mor: the 64 haploid spermatids (spt) undergo dramatic morphological changes. Only 6 elongating spermatids are shown for clarity. Because of the length of the sperm tail, fully elongated spermatids have their nucleus at the terminal end of the testis, while the tail extends almost to the apical end. During this last stage, the two cyst cells become structurally distinct, the head-cyst cell (cyh) being associated with the sperm heads and the tail-cyst cell (cyc) elongating with the growing sperm tails. The head-cyst cell then becomes entrapped by a specialized epithelial cell (tec) located in the terminal part of the testis. Coiling of the sperm bundle ensues, followed by release of motile spermatozoa (spz) into the seminal vesicle. Only one spermatozoon is shown for clarity. See text for additional information.
expected for cyst cells. The identity of these cells was further ascertained by showing that the β-galactosidase-positive nuclei (Fig. 4C, arrows) were located within cytoplasm containing β3-tubulin (Fig. 4D), a β-tubulin isotype specifically expressed in cyst cells in male gonads (Kimble et al., 1989).

Several lines labeled cyst cells in a stage-specific manner. For instance, some labeled mainly early cyst cells (Fig. 5A), including cyst progenitor cells (Fig. 5A, arrows). As expected from the ultrastructural data, cyst cell nuclei were smaller and not as spherical as neighbouring germ line stem cells (Fig. 5A, filled arrowhead), and were located further away from the apical cells of the hub (Fig. 5A, outlined arrowhead). Other lines labeled cyst cells associated with late proliferative and early growth phase germ cells, but not later stages of differentiation (Fig. 5B, arrows). These transient expression patterns indicate that cyst cells adopt distinct identities in the period prior to meiosis.

Some lines labeled cyst cells only in postmeiotic stages of the differentiation program (Fig. 5C, arrows), an observation confirmed by the lack of staining in larval gonads (data not shown), where only premeiotic stages are represented. A few lines specifically labeled tail-cyst cells but not head-cyst cells (Fig. 5D, arrows). The tail-cyst cell nuclei could be easily recognized by their invariant location, squeezed against the periphery of elongated sperm tail bundles (Fig. 5E). One line expressed lacZ in both tail- and head-cyst cells. The head-cyst cell nuclei could be identified by their position at the base of the testis and their close association with sperm heads (Fig. 5F, arrowheads and corresponding arrows).

Apical cells of the hub

At the apical tip of the testis, a group of 12 to 16 small somatic cells form the hub around which stem cells are anchored (Hardy et al., 1979; Fig. 1B, hub). We identified marker lines that express lacZ either exclusively in these apical cells (Fig. 6A, arrow) or in these apical cells as well as in early cyst cells (Fig. 5A). The identity of the labeled cells was suggested by their clustering (Fig. 6B, arrow) and by the radial position of germ line stem cells (Fig. 6B, arrowhead) around the cluster. We first confirmed that the lacZ-expressing cells are derived from somatic and not germ line lineage by crossing these marker lines to osk- females (Lehman and Nüsslein-Volhard, 1986). Progeny resulting from such a cross lack germ cells but retain some somatic components of the gonads, including the apical cells of the hub (Aboim, 1945). β-galactosidase remained present in such an agamic gonad (Fig. 6C, arrow). We also noted that the hub in the agamic gonad was both larger and positioned some distance away from the apical tip as compared to the normal testis (Fig. 6C, arrowhead; see Discussion). Additional evidence that the labeled cells indeed constitute the apical hub was obtained by showing that the cluster of β-galactosidase positive nuclei (Fig. 6E, arrow) also expressed fasciclin III (Fig. 6F, arrow), a marker specific for the apical cells of the hub in male gonads (Brower et al., 1981).

By morphological criteria, the apical cells can be identified as early as the first instar larval gonad (Aboim, 1945). We examined one line at this developmental stage to determine whether it was a faithful marker for these cells. Indeed, we observed strong lacZ expression in the apical cells of the first instar larval gonad (data not shown). Furthermore, in 13 to 15 hour embryos, a cap of β-galactosidase positive cells was observed at the apical side of the newly formed gonad (Fig. 6G, arrow). Thus, this line represents an early molecular marker for the presumptive apical cells in the gonadal mesoderm.

Terminal epithelium

At the terminal end of the male gonad, the testis sheath includes a layer of epithelial cells that fuse with the seminal vesicle. We identified marker lines that label most epithelial cells of the terminal region (Fig. 7A, ...
Fig. 5. Particular lines label cyst cells in a stage-specific manner. (A) Apical region, showing labeling of early cyst cells, including cyst progenitor cells (arrows). Note a neighboring unlabeled germ line stem cell (solid arrowhead) and the labeled apical cells of the hub (outlined arrowhead) (line 842). This line also labels pigment cells, terminal epithelial cells and head-cyst cells (not shown here). (B) Apical region, showing transient labeling of cyst cells in the late proliferative and early growth phase regions (arrows point at the youngest and oldest labeled cyst cells, respectively) (line 901). (C) Adult testis shows labeling of cyst cells postmeiotically only (arrows). Head-cyst cells are indicated by a solid arrowhead (line ms-985; this is a male-sterile line, stained here as a heterozygote). This line also labels germ line stem cells (not visible at this magnification) and terminal epithelial cells (outlined arrowhead). (D, E) Tail-cyst cells (line 498). (D) Adult testis, showing labeling of tail-cyst cells only (arrows). (E) Tail-cyst cell nucleus between two elongated sperm tail bundles. (F) Head-cyst cells. Immunoperoxidase staining of the terminal region of an adult testis with anti-β-galactosidase antibody. Hoechst counterstain shows the association of rod-shaped sperm head nuclei (arrows) with the β-galactosidase positive head-cyst cell nuclei (arrowheads) (line 57; this line displayed unusually high levels of endogenous β-galactosidase activity which necessitated analysis using the antibody directed against bacterial β-galactosidase). Bars=50 µm.
Fig. 6. Marker line labeling the apical cells of the hub (line 254; this line is homozygous lethal and therefore examined as a heterozygote). (A) Adult testis, showing labeling of the hub located at the apical tip of the testis (arrow). There is also non-specific staining in degenerating cysts (solid arrowhead) and waste bags (outlined arrowhead). (B) Apical-most portion, showing labeling of the clustered somatic cells of the hub (arrow) and radially disposed unlabeled germ line stem cells (arrowhead) anchored around the hub. (C) Immunoperoxidase staining of an agamic testis, demonstrating the somatic nature of the β-galactosidase positive cells. The hub in the agamic testis (arrow) is larger and positioned further away from the apical tip as compared to the normal testis (arrowhead); note that this line also labels cells from the seminal vesicle sheath (outlined arrowhead). (D,E,F) Identification of the β-galactosidase positive cells as the apical cells of the hub. (D) Hoechst counterstain revealing all nuclei present at the apical tip of the testis, viewed from above. (E) Same apical tip as in D labeled with β-galactosidase antibody visualized by indirect immunofluorescence; note the clustering of the labeled cells (arrow). (F) Same apical tip as in D and E labeled with fasciclin III antibody visualized by indirect immunofluorescence; note that the labeled cells (arrow) are the same as those positive for β-galactosidase (compare arrows in D and E). (G) Immunoperoxidase staining with anti-β-galactosidase antibody of a 13-15 hour embryonic gonad. The gonad was dissected away from the embryo to highlight the labeling observed in the apical portion of the gonad (arrow); note the prominent pole cells (arrowhead). Other cell types also stain during embryogenesis (M. Whiteley et al., 1992). Bars=50 μm.
Fig. 7. Marker lines label overlapping subsets of cells in the terminal epithelium. (A,B) Most or all terminal epithelial cells (line 34). (A) Adult testis; staining begins in a region where coiling of sperm bundles occurs (arrow) and extends down to the junction with the seminal vesicle (arrowhead). This line also weakly labels cyst cells in the late proliferative and early growth phase regions (not visible in this focal plane) (B) Terminal region, showing that the labeled cells (arrows) are part of the sheath of the testis rather than the lumen (arrowhead). (C) Terminal region, showing that only a subset of epithelial cells are labeled (solid arrows), probably those that entrap the head-cyst cells (line 429). Note the lack of staining in the more terminal epithelial cells (outlined arrowheads), near the junction with the seminal vesicle (solid arrowhead); note also the presence of coiling sperm bundles (outlined arrow) in the area where the labeled cells are located. Bars=50 μm.
from the arrow to the arrowhead). The labeled cells were identified by the fact that the stain reaches the seminal vesicle (Fig. 7A, arrowhead) and on the location of the labeled nuclei within the sheath of the testis (Fig. 7B, arrows), rather than the lumen (Fig. 7B, arrowhead).

One line specifically labeled what appears to be the specialized epithelial cells that entrap the head-cyst cells prior to the coiling of spermatids (Tokuyasu et al., 1972, Fig. 1B, tec). We infer this because the labeled cells are a subset of the terminal epithelial cells (Fig. 7C, solid arrows, compare with Fig. 7B) and are located in an area with many coiled sperm bundles (Fig. 7C, outlined arrow).

Other cells of somatic origin found in testes include pigment and muscle cells from the sheath. We identified marker lines labeling each of these cell types in conjunction with other cell types (data not shown).

**Discussion**

Little is known about the genes governing the differentiation of germ line and somatic cells during spermatogenesis in *Drosophila melanogaster*. Given the limitations of a classical mutant analysis, we sought to study this developmental pathway using a complementary approach. We have examined over 700 homozygous viable and fertile enhancer detector lines for *lacZ* expression in male gonads. We have described staining patterns for the different cell types present in testes, denning in some cases stages that had not been recognized previously by morphological criteria. A summary of the marker lines described in this report is given in Table 1.

**Expression in the germ line**

β-galactosidase activity is found in the male germ line in 68% of the lines examined, a figure that is more than double that reported in two similar studies conducted with oogenesis (Fasano and Kerridge, 1988; Grossniklaus et al., 1989). This could simply be indicative of a larger number of cis-acting elements active during male versus female gametogenesis. More probably, this could be due to the fact that the lines were generated by a transposition event occurring in the male germ line. Indeed, it has been shown that transposition events occurring in the male germ line lead to a higher frequency of *lacZ* expression in male than in female germ cells, the reverse being the case when the transposition event occurs in the female germ line (Bownes, 1990).

Most lines that stain germ cells start expressing *lacZ* in the growth phase. This is consistent with uridine incorporation experiments which showed that the bulk of transcription in the germ line occurs during this phase (Olivieri and Olivieri, 1965; Gould-Somero and Holland, 1974). Those studies also led to the postulate that transcription ceases prior to meiosis during *Drosophila* spermatogenesis. Accordingly, we have not identified any line in which *lacZ* expression occurs strictly postmeiotically in the germ line. Such strict postmeiotic transcription units may yet exist, but not be represented in a collection of viable and fertile lines. Alternatively, such loci may be refractory to P-element insertion and be under-represented among enhancer detector lines.

**Male germ cell identity**

Two marker lines express *lacZ* in the proliferative phase of male but not female gametogenesis, characterizing them as markers of early male germ cell identity (Fig. 3A and 3B and data not shown). Recent work has

**Table 1. Summary of the marker lines described in this report.** Enhancer detector lines were obtained from the laboratories of Y.-N. and L. Jan (a), M. Fuller and M. Scott (b), J. Kassis (c), and J. Merriam (d); see Materials and methods

<table>
<thead>
<tr>
<th>Line and origin</th>
<th>Cytogenetic location*</th>
<th>Staining pattern in testes</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 (a)</td>
<td>62A/B</td>
<td>All terminal epithelial cells; cyst cells in late proliferative and early growth regions.</td>
<td>7A and B</td>
</tr>
<tr>
<td>57 (a)</td>
<td>82C</td>
<td>Head- and tail-cyst cells.</td>
<td>5F</td>
</tr>
<tr>
<td>254 (c)</td>
<td>35C</td>
<td>Apical cells of the hub.</td>
<td>6A through G</td>
</tr>
<tr>
<td>420 (a)</td>
<td>88E</td>
<td>Subset of terminal epithelial cells.</td>
<td>7C</td>
</tr>
<tr>
<td>498 (a)</td>
<td>24A</td>
<td>Tail-cyst cells.</td>
<td>5D and E</td>
</tr>
<tr>
<td>600 (a)</td>
<td>21F/22A</td>
<td>Cyst cells, starting in the growth region; apical cells of the hub; terminal epithelial cells; pigment cells.</td>
<td>4A through D</td>
</tr>
<tr>
<td>606 (a)</td>
<td>28C</td>
<td>Male but not female germ line stem and gonial cells; tail-cyst cells; terminal epithelial cells.</td>
<td>3A and B</td>
</tr>
<tr>
<td>817 (b)</td>
<td>(ND)</td>
<td>Germ cells, starting in the growth phase.</td>
<td>2A through D</td>
</tr>
<tr>
<td>842 (b)</td>
<td>57F</td>
<td>Early cyst cells, including cyst-progenitor cells; apical cells of the hub; head-cyst cells; terminal epithelial cells; pigment cells.</td>
<td>5A</td>
</tr>
<tr>
<td>901 (b)</td>
<td>92B</td>
<td>Cyst cells in late proliferative and early growth regions.</td>
<td>5B</td>
</tr>
<tr>
<td>ms-985 (d)</td>
<td>28C</td>
<td>Cyst cells in postmeiotic aspects; terminal epithelial cells; germ line stem cells.</td>
<td>5C</td>
</tr>
</tbody>
</table>

*(ND) – not determined.*
begun to unravel the sex-determination pathway in the male and female germ line (Steiman-Zwicky et al., 1989; reviewed by Pauli and Mahowald, 1990). In females with mutations that interfere with this pathway, ovaries are filled with small cells that resemble germ cells embarked on a male differentiation program. The sexual identity of such cells can sometimes be ambiguous when defined solely by morphological criteria. Markers of early male germ cell identity would, therefore, be useful in ascertaining the sexual nature of germ cells in mutants putatively affected in germ line sex-determination. The usefulness of our marker lines was tested by crossing them to sans-fille (snf), a well-characterized germ line sex-determination mutant (Oliver et al., 1988). In a snf mutant female, XX germ cells now express β-galactosidase (D. Pauli and A.P. Mahowald, personal communication). This confirms the transformed sexual identity of XX germ cells in a snf mutant and demonstrates that these two marker lines can indeed serve to probe early male germ cell identity.

**Cyst cell differentiation**

We have shown that most lines that stain cyst cells label only a subset of the cyst cells present during the course of spermatogenesis. For the period prior to meiosis, we find at least three distinct types of staining patterns in cyst cells. The first type labels early cyst cells, including cyst progenitor cells and shows little or no expression in later stages of cyst cell differentiation (Fig. 5A). The second type labels cyst cells exclusively in the late proliferative and early growth phase regions (Fig. 5B). The third and most common type labels cyst cells starting in the growth phase region and most or all of the cyst cells in the period after meiosis (Fig. 4A, B). These distinct types of staining patterns suggest that there are different stages in cyst cell differentiation in the period prior to meiosis. Such distinctions had not been detected by morphological criteria.

After meiosis occurs in germ cells, the two cyst cells become structurally distinct, one being associated with the sperm heads and the other with the elongating sperm tails. Accordingly, we found lines that label either only the tail-cyst cells (Fig. 5D, E) or both tail- and head-cyst cells (Fig. 5F). Thus, cyst cell differentiation for the period both before and after meiosis can be broken down into a succession of stages revealed by our marker lines.

**Interaction between germ line and soma**

In oogenesis, cooperation between germ line and soma is crucial for proper development of the egg (Schüpbach, 1987). It is not yet known whether a similar requirement has to be met for correct sperm development. However, recent experiments in germ line sex determination are consistent with some interaction taking place in male gametogenesis as well. When transplanted into a female host, chromosomally male germ cells embark on the spermatogenic differentiation program but arrest at the spermatocyte stage, possibly because they need a compatible soma to differentiate further (Steiman-Zwicky et al., 1989).

The marker lines described here can probe the extent to which germ line and soma cooperate during the course of spermatogenesis. For example, the lines that reveal transient cyst cell identities will allow us to monitor the progression of cyst cell fate in mutant backgrounds where the differentiation of the germ cells is blocked.

Agametic gonads provide an experimental situation in which to address the role of the germ line in directing specific somatic cell fates. We have shown that the apical cells of the hub around which stem cells are anchored are still present in agametic gonads (Fig. 6C). Based on morphological criteria alone, it had been concluded that the hub is not altered in agametic gonads (Aboim, 1945). However, the hub in an agametic environment appears different in two respects. First, it is larger than in normal testes, due to an increase in cell size, rather than cell number (P.G. and S.D., unpublished data). Second, the hub is displaced from the very apical tip of the agametic testis. We have not yet determined the cause of this displacement, nor the nature of the cells occupying the apical-most position. Taken together, our observations confirm that the apical cells can organize into a hub in the absence of germ line and suggest that the germ cells plays some role, direct or indirect, in controlling the size of the somatic hub and its position in the testis.

**Gonadogenesis**

The gonad is formed at about 12 hours during embryogenesis, when the pole cells become surrounded by gonadal mesoderm (Sonnenblick, 1941; Aboim, 1945; Hay et al., 1988). All morphologically distinct somatic cells of the adult testis derive from this common mesodermal primordium. Marker lines that label specific somatic cells early in development are instrumental in addressing when these different cell types assume their fate. We have shown that a marker line labeling the apical cells of the hub in the adult testis represents an early molecular marker for the presumptive apical cells in the embryonic gonadal mesoderm (Fig. 6G). Several marker lines that identify the other somatic cell types in testes also label presumptive cells in the embryonic gonad (M. Boyle, P.G., SV. and S.D., unpublished data). Studies using such marker lines can unravel the origin and lineage of the several somatic components of male gonads.

**Probing spermatogenesis with enhancer detectors**

We have discussed how marker lines are instrumental in investigating gonadogenesis and in revealing the extent of interaction between germ line and soma during spermatogenesis. In addition, this collection of marker lines should allow the characterization of genes expressed at critical times and places during male gametogenesis. Together with an analysis of enhancer detector male-sterile mutants, these approaches should help in defining key regulators of this developmental pathway.

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Margaret Fuller and Matthew Scott, Judy Kassis, John Merriam for generously providing the enhancer detector lines used in this study. We thank Margaret Fuller also for fruitful discussions and constant encouragement. We are grateful to Danny Brower, Alfonso Martinez-Arias and Elizabeth Raff for their gift of antibodies. We benefited from the efficient help of Carrie Bromleigh in determining the cytogenetic location of the P-element in the marker lines. We are indebted to Peter Bokor, Monica Boyle, Claude Desplan, Scott Dougan, Laurent Fasano, Marianna Giarré, Janet Mullen and Elelta Ronchi for careful reading of the manuscript. All members of the DiNardo and Desplan laboratories are acknowledged for providing an exciting scientific environment. We wish to thank Sam Ward for encouraging us to initiate a molecular genetic analysis of spermatogenesis in D. melanogaster. S.D. would also like to thank Allan Spradling and members of his laboratory for an early and stimulating discussion about the enhancer detector patterns they were obtaining in ovaries. S.D. is a Lucille P. Markey Scholar and for their gift of antibodies. We benefited from the efficient help of Carrie Bromleigh in determining the cytogenetic location of the P-element in the marker lines. We are indebted to Peter Bokor, Monica Boyle, Claude Desplan, Scott Dougan, Laurent Fasano, Marianna Giarré, Janet Mullen and Elelta Ronchi for careful reading of the manuscript. All members of the DiNardo and Desplan laboratories are acknowledged for providing an exciting scientific environment. We wish to thank Sam Ward for encouraging us to initiate a molecular genetic analysis of spermatogenesis in D. melanogaster. 

NOTE

All marker lines mentioned in this report are available from the authors upon request.

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